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Nitric Oxide-Releasing Calcium Channel Modulators and Non-steroidal Antiinflammatory Drugs Possessing a N-substituted Diazen-1-ium-1,2-diolate Moiety

by

©

Carlos Alberto Velázquez Martínez

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This work is dedicated to:

My father, Ramon Velazquez (1946-2002) who died of Congestive Heart Failure, while I was working to find a pharmacological alternative against this silent but terrible disease.

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Abstract

Two distinct classes of hybrid nitric oxide (•NO)-releasing drugs were synthesized and evaluated using *in vitro* and/or *in vivo* assays. A group of hybrid •NO-releasing calcium channel (CC) modulators (**67-80**) were designed for use in the potential treatment of congestive heart failure. Hybrid •NO-releasing non-steroidal antiinflammatory drugs (•NO-NSAIDs) were designed for evaluation as selective (**81-88**) or non-selective (**89-94**) cyclooxygenase-2 (COX-2) inhibitors.

Racemic 4-aryl(heteroaryl)-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylates, possessing a potential •NO donor diazen-1-ium-1,2-diolate (**67-80**) ester substituent, exhibited a weaker smooth muscle CC antagonist activity, relative to the reference drug nifedipine. Compounds **74** and **79** constitute novel •NO donor compounds with *dual cardioselective agonist/smooth muscle selective antagonist activities*, in conjunction with an *enhanced nitric oxide release profile*.

A group of 3,4-diphenyl-1,2,5-oxadiazole-2-oxides (**81-84**), and 3,4-diphenyl-1,2,5-oxadiazoles (**85-88**), were designed as hybrid •NO-releasing selective cyclooxygenase-2 inhibitors. Regioisomers **82a-82b**, like the reference drug celecoxib, were potent *in vitro* COX-2 inhibitors. Docking studies showed that the methanesulfonyl COX-2 pharmacophore present in regioisomers **82a-82b** is positioned in the vicinity of the COX-2 secondary pocket. The release of •NO from compounds **81-84** was thiol-dependent. These results suggest that the 1,2,5-oxadiazole-2-oxide ring possesses beneficial features that should be present in a central ring template, pertinent to the design of novel hybrid •NO-releasing COX-2 inhibitor agents that may be free from adverse cardiovascular effects.

Finally, a group of •NO-NSAIDs possessing a diazen-1-ium-1,2-diolate moiety (**89-94**), were designed as non-ulcerogenic NSAIDs. Although none of these •NO-NSAIDs exhibited *in vitro* cyclooxygenase inhibitory activity,

compounds **89-94** showed equipotent antiinflammatory activities *in vivo* to that of the parent compounds aspirin, ibuprofen and indomethacin. Prodrugs **89-94** released •NO upon incubation with either phosphate buffer at pH 7.4, or porcine liver esterase, but the percentage •NO released was up to 6-fold higher upon incubation with guinea pig serum. Compounds **89-92** produced no observable lesions in an ulcer index assay. The simultaneous release of aspirin and •NO from compounds **89** and **90** constitutes a potentially beneficial property for the prophylactic prevention of thrombus formation and adverse cardiovascular events such as stroke and myocardial infarction. Accordingly, these hybrid •NO-NSAID prodrugs possessing a diazen-1-ium-1,2-diolate moiety, represents a new approach for the rational design of antiinflammatory drugs with reduced gastric ulcerogenicity.

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LIST OF ABBREVIATIONS

δ	In an NMR context, "δ" denotes "chemical shift" expressed in parts-per-million (ppm).
Anal. Calcd.	Microanalytical calculations
Arg	Arginine
CCags	Calcium channel agonist(s)
CCants	Calcium channel antagonist(s)
C-2	Position C-2 of the 1,4-dihydropyridine ring
C-3	Position C-3 of the 1,4-dihydropyridine ring
C-4	Position C-4 of the 1,4-dihydropyridine ring
C-5	Position C-5 of the 1,4-dihydropyridine ring
C-6	Position C-6 of the 1,4-dihydropyridine ring
$CDCl_3$	Deuterated chloroform
conc.	Concentrated
cGMP	Cyclic guanosine monophosphate
d	In a spin multiplet context, "d" denotes "doublet".
dd	In a spin multiplet context, "dd" denotes "doublet of doublets".
DCM	Dichloromethane
DHP	Dihydropyridine
DMAP	4-(*N*,*N*-Dimethylamino)pyridine
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethyl sulfoxide
DMSO-d_6	Deuterated dimethyl sulfoxide
D_2O	Deuterated water
EC_{50}	50% Effective Concentration. The concentration of a compound that is required to produce 50% of the maximum effect.
Et	Ethyl
ether	Diethyl ether
EtOAc	Ethyl acetate

EtOH	Ethanol
FMN/NADH	Flavin mononucleotide / reduced β-nicotinamide-adenine dinucleotide
Gln	Glutamine
h	Hours
Ile	Isoleucine
His	Histidine
HMPA	Hexamethylphosphoramide
^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
HPLC	High performance liquid chromatography
IC_{50}	50% Inhibitory Concentration. The concentration of a compound that is required to produce 50% inhibition.
IR	Infrared spectroscopy
J	Vicinal coupling constant (expressed in Hertz)
L-NMMA	N^G-monomethyl-L-arginine
m	In a spin multiplet context, "m" denotes "multiplet".
Me	Methyl
MeCN	Acetonitrile
MeOH	Methanol
min	Minute
mp	Melting point (°C)
MsCl	Methanesulfonyl chloride
n	In a statistical context, "n" denotes "the number of test samples".
NADPH	The reduced form of β-nicotinamide-adenine dinucleotide phosphate
NaOMe	Sodium methoxide
n-Bu	Normal-butyl
N-1	Position N-1 of the 1,4-dihydropyridine ring
NMR	Nuclear magnetic resonance spectroscopy
•NO	Nitric oxide
NOS	Nitric oxide synthase

eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
OAc	Acetoxy
Ph	Phenyl
Phe	Phenylalanine
psi	Pound per square inch
q	In a spin multiplet context, “q” denotes “quartet”.
s	In a spin multiplet context, “s” denotes “singlet”.
sGC	Soluble guanylate cyclase
SEM	Standard error of the mean
S_N2	Nucleophilic substitution type 2
t	In a spin multiplet context, “t” denotes “triplet”.
t-Bu	Tertiary-butyl
THF	Tetrahydrofuran
Tyr	Tyrosine
TLC	Thin layer chromatography
v/v	Volume per volume mixture
w/w	Weight per weight mixture

1.0. Introduction

1.1. Coronary artery disease (CAD)

Worldwide, one third of all deaths each year are due to cardiovascular diseases, including 7 million heart attacks and 5 million strokes. Coronary artery disease (CAD) is the single most important cause of death and, more importantly, the single major cause of premature deaths in modern, industrialized countries. CAD covers a wide spectrum of diseases including acute coronary syndromes, myocardial infarction, and ischaemic cardiomyopathy with chronic heart failure. The spectrum of diseases encompassed by CAD has a common pathology: coronary artery narrowing as a consequence of ATHEROSCLEROSIS*, a focal, dynamic pathological process, involving inflammatory fibroproliferative responses to multiple forms of endothelial injury.[1]

CAD (sometimes referred to as coronary heart disease) is characterized by different extents of clogging of the vessels that carry blood and oxygen to the heart muscle (coronary arteries) with atherosclerotic plaque. In advanced disease states, partially blocked arteries can prevent the heart from getting sufficient blood and oxygen, causing chest pain (angina). If a blood clot forms, it can suddenly cut off blood flow in the artery and cause a heart attack.[2]

1.2. Congestive heart failure (CHF)

Despite a steady decline in morbidity from CAD during the past 25 years, cardiovascular diseases remain the leading cause of mortality in Canada accounting for 37% of total deaths.[3] Congestive heart failure (CHF), which is defined as the inability of the heart to expel sufficient blood to keep pace with the metabolic demands of the body, develops in 50–60% of patients with CAD, valvular insufficiency, and rheumatic heart disease. CHF is a growing public health problem for most countries around the world. There are currently more than 10 million diagnosed CHF patients in North America and Western Europe.[4] It is estimated that there are 400,000 new cases of CHF each year.[5] According to a recent collaboration study reported by Cujec and coworkers, there were a total of 32,139 hospital admissions due to CHF in the province of Alberta from 1994 to 2000.[6]

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* ATHEROSCLEROSIS: arterial disease in which raised areas of degeneration and cholesterol deposits form on the inner surfaces of the arteries

CHF originates as a direct result of MYOCARDIAL INJURY[*] which causes left ventricular dysfunction. This activates a variety of peripheral vasomotor mechanisms that are both beneficial (compensatory) and deleterious. Compensatory mechanisms may sustain cardiac function and tissue perfusion for a while, but over time and with increasing cardiac workload, the compensatory mechanism become a self-destructive perpetuating loop of increasing cardiac failure. Increased retention of sodium and water causes further ventricular distension causing decreased ventricular contraction. This then causes greater hypertrophy and increased heart rate (HR). Increased HR increases myocardial oxygen demand and decreases diastolic filling time leading to myocardial ischemia and decreased cardiac output, respectively. The cycle then starts over again causing greater damage and increased severity of cardiac failure.[7]

1.3. Drugs used for the treatment of CHF and angina

Patients are generally managed using a complex regimen of medications and requirements for lifestyle change, and often require numerous admissions to hospital. Recent advances in therapeutic interventions have resulted in impressive age-adjusted declines in the incidence and hospital mortality of cardiovascular disease; however, the prognosis of CHF patients remains grim, with a 5-year survival rate usually lower than 50%.[8]

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[*] MYOCARDIAL INJURY: tissue damage produced in the heart muscle (myocardium).

Although each particular case must be clinically evaluated, the general approach for the treatment of CHF may include:

- Inotropic drugs → Increase cardiac contractility
- Diuretics → Decrease PRELOAD[*]
- Vasodilators, ACE inhibitors → Decrease AFTERLOAD[†]
- Calcium channel blockers → Normalize heart rate

1.3.1. Inotropics. Cardiac glycosides.

Cardiac glycosides are an important class of naturally occurring drugs (produced mainly in plants such as *Digitalis purpurea*, *Digitalis lanata*, foxglove plant, *Strophanthus gratus*, and *Strophanthus kombe*) whose actions include both beneficial and toxic effects on the heart. These natural products contain a sugar and an aglycone (nonsugar) moiety. Based on the nature and number of sugar molecules, as well as the number of hydroxyl groups on the aglycone moiety, each glycoside assumes different generic names. The aglycone portion of the cardiac glycosides is a steroid nucleus with a unique set of fused rings (Figure 1).[9]

The most widely accepted mechanism of action of cardiac glycosides, is their ability to inhibit the membrane bound Na$^+$, K$^+$-adenosine triphosphatase (Na$^+$, K$^+$-ATPase) pump responsible for sodium/potassium exchange. To explain the correlation between this ion pump and the mechanism of action of cardiac glycosides on the heart muscle contraction, it is necessary to consider the biochemical events associated with cardiac action potentials. Human cells have a characteristic profile of ion gradients across the plasma membrane under steady-state conditions. In this regard, the intracellular Na^+ concentration is relatively low, whereas the intracellular K^+ concentration is relatively high, compared to the extracellular concentrations of these ions. This specific condition requires energy and *is maintained by the action of the* Na^+*,* K^+*-ATPase*, which accounts for 25% or more of the basal energy metabolism of a typical cell. Because cardiac glycosides inhibit this enzyme, they produce an increased concentration of intracellular Na^+. Elevated intracellular Na^+ triggers the influx of calcium (Ca^{2+}), which in turn, results in efflux of K^+ out of myocardial cells. Elevated intracellular Ca^{2+} concentration initiates a series of biochemical events (see section 1.3.4.1.1) that eventually induce an increase in the force of myocardial contraction.[9]

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[*] PRELOAD: it is the left ventricular filling pressure. Normally, the amount of blood filling the heart is directly proportional to the pressure created by the myocardial fiber stretch.

[†] AFTERLOAD: it is the pressure the ventricle must work against to pump blood out of the heart.

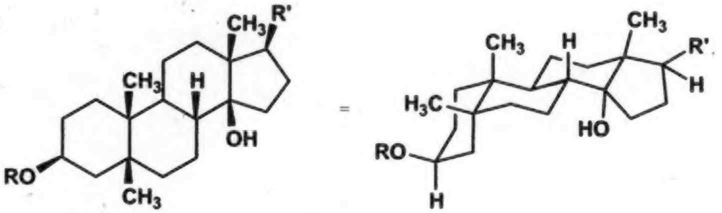

Figure 1: Basic structure of cardiac glycosides. R = monosaccharide or polysaccharide with a β-1,4-glucosidic linkage; R' = five or six membered ring lactone, which contains one or two unsaturations.

Digoxin (Figure 2) is the most widely used cardiac glycoside. The INOTROPIC EFFECT[*] of digoxin on myocardium is dose-dependent. In patients with heart failure, increased contractile force boosts cardiac output, and improves systolic emptying. Increased myocardial contractility and cardiac output reflexively reduce sympathetic tone. This compensates for the direct vasoconstrictive action of the drug, thereby reducing total peripheral resistance.[10]

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[*] INOTROPIC EFFECT: biochemical mechanism by which a drug increases the force of myocardial contraction.

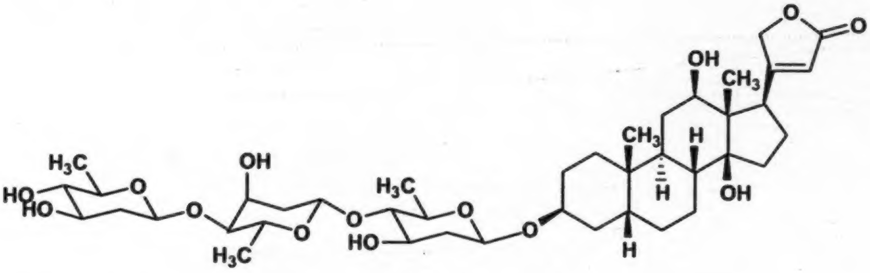

Figure 2: Chemical structure of digoxin (a cardiac glycoside).

Although cardiac glycosides have been used for over 200 years, they remain controversial agents in the treatment of heart failure. The Digitalis Investigation Group showed that the use of digitalis preparations improved symptoms and functional capacity and led to a reduction in the rate of admissions to hospital, however, there was no change on mortality rates.[11] All cardiac glycosides preparations have the potential to cause toxicity. Because the minimal toxic dose of glycosides is only two or three times the therapeutic dose (narrow therapeutic window) intoxication is common. The high levels of Ca^{2+} are responsible for the observed cardiac arrhythmias characteristic of cardiac glycoside treatments.[9]

1.3.2. Diuretics.

Diuretics are drugs that increase the rate of urine formation. By increasing the urine flow rate, diuretic usage leads to the increased excretion of electrolytes (especially sodium and chloride ions) and water from the body. Their pharmacological properties have led to the use of diuretics in the treatment of edematous conditions resulting from a wide variety of causes, including congestive heart failure. These drugs should be prescribed for all patients with symptoms of heart failure who have a predilection to fluid retention. Diuretics are effective in relieving the symptoms and signs of fluid retention (edema). The Randomized Aldactone Evaluation Study of patients with heart failure showed a reduction in both cardiac death and admissions to hospital for deteriorating heart failure in patients receiving diuretic therapy.[12]

1.3.3. Angiotensin-converting enzyme (ACE) inhibitors.

ACE inhibitors prevent the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor. Inhibition of angiotensin II production decreases vasoconstriction and reduces peripheral arterial resistance. Adrenocortical aldosterone secretion is also reduced, which results in decreased sodium and water retention (decreased extracellular fluid volume).[13] Therefore, ACE inhibitors are used primarily to treat hypertension due to increased plasma volume. All patients with heart failure produced by left ventricular systolic dysfunction should receive an ACE inhibitor, unless they are intolerant of the drug or have a contraindication to its use. Several landmark studies with ACE inhibitors (CONSENSUS, SOLVD, SAVE) showed the benefit of treating peripheral vasoconstriction and heart failure by inhibiting the rate-limiting enzyme (ACE) in the renin–angiotensin system.[14] Total mortality, admissions to hospital, worsening heart failure and recurrent myocardial infarctions are reduced by 20-25%.[15]

1.3.4. Calcium channel (CC) modulators

Calcium channel modulators are drugs that control or modulate the movement of Ca^{2+} ions across cell membranes. There are two different types of CC modulators:

I. Calcium channel ANTAGONISTS[*][16] (CCants): also called calcium channel blockers, are compounds that prevent the entry of Ca^{2+} into cells.

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[*] ANTAGONIST: it is defined as a drug or an endogenous substance that opposes the physiological effects of another. At the receptor level, an antagonist is a chemical entity that opposes the receptor-associated responses normally induced by another bioactive agent.

II. Calcium channel AGONISTS*[16] (CCags): are compounds that enhance or promote the entry of Ca^{2+} into cells.

There are three major classes of CC antagonists (Figure 3) currently approved for the treatment of angina:

- 1,4-dihydropyridines (1,4-DHPs): nifedipine, nicardipine, and amlodipine
- Benzothiazepine derivatives: diltiazem
- Alkyl aryl amines: verapamil and bepridil

1.3.4.1. Dihydropyridines (1,4-DHPs)

A wide variety of heterocyclic 1,4-dihydropyridines (1,4-DHPs) have been reported as cardiovascular agents.[17-26] Nifedipine (**1**, Figure 3) was the first 1,4-DHP to be used clinically. It blocks the entry of Ca^{2+} ions through L-type potential-dependent CCs, primarily in the peripheral vasculature. Therefore, it is a potent vasodilator that selectively dilates arterial resistance vessels at a lower concentration than that needed to induce negative inotropic effect.[27,28] Therefore, following a dose of nifedipine, the overall effects are lowering of blood pressure and a modest increase in both heart rate and cardiac output.[29] However, despite its commercial success in clinical use, there are a number of side effects associated with nifedipine administration. Most of these side effects are caused primarily by excessive peripheral vasodilation, which causes dizziness, flushing, headache and palpitations.[30] Adverse effects including systemic hypotension, cardiogenic shock, pulmonary edema and even death have also been reported.[31]

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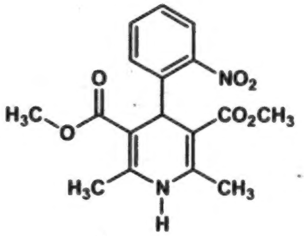
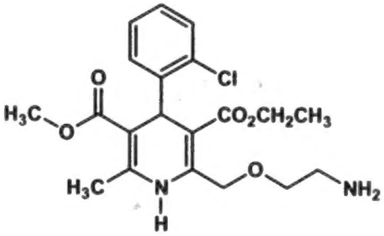
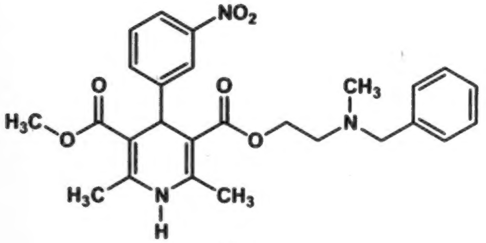
[*] AGONIST: it is defined as a drug or endogenous substance that can interact with a receptor and initiate a pharmacological response, characteristic of that receptor (contraction, secretion, etc.).



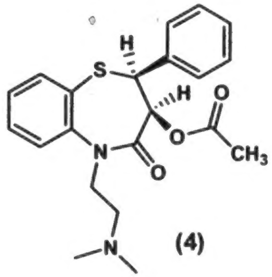
(1)



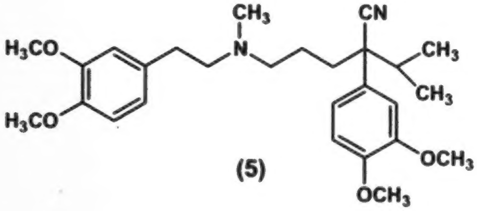
(2)



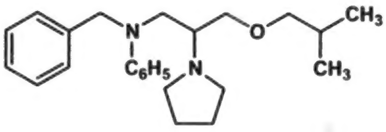
(3)



(4)



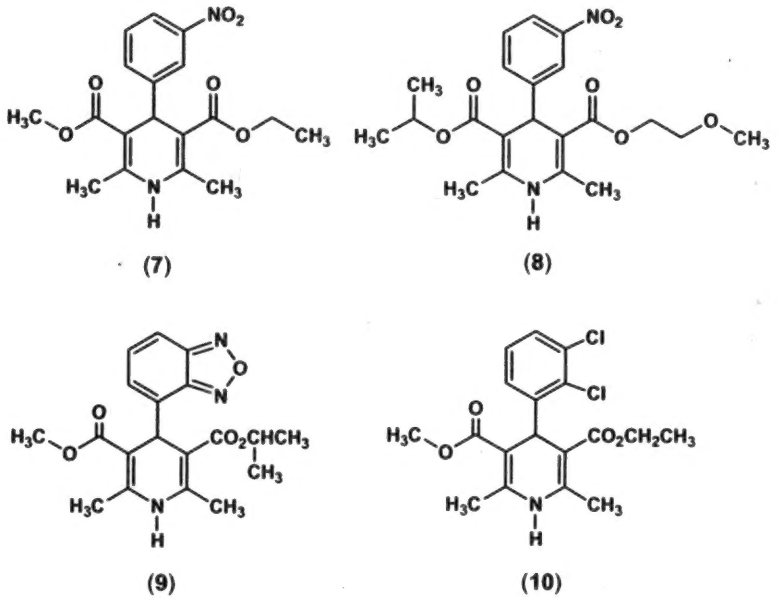
(5)



(6)

Figure 3: Chemical structures of different calcium channel blockers. Nifedipine (**1**), amlodipine (**2**), nicardipine (**3**), diltiazem (**4**), verapamil (**5**), bepridil (**6**).

The commercial success of nifedipine stimulated an array of structure-activity studies in an effort to obtain analogs with higher potencies, a longer duration of action and less side effects.[32] Some of these second-generation calcium channel antagonists include nicardipine (**3**, Figure 3), nitrendipine (**7**), nimodipine (**8**), isradipine(**9**) and felodipine (**10**) illustrated in Figure 4.[33-35]


(7) (8)

(9) (10)

Figure 4: Chemical structures of different 1,4-DHP CC blockers. Nitrendipine (**7**), nimodipine (**8**), isradipine (**9**), and felodipine (**10**).

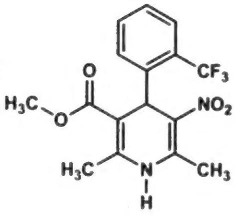
Nicardipine (**3**) which is the most potent of the 1,4-DHPs studied for their effect on vascular smooth muscle, also exhibits the highest degree of selectivity for vascular smooth muscle relaxation, and it shows fewer side effects compared to nifedipine.[36]

Nitrendipine (**7**) exhibits a longer duration of action than nifedipine and has high selectivity for vascular smooth muscle. Because it does not exhibit negative inotropic effects, nitrendipine can be administered to patients with CHF and those with hypertension complicated by left ventricular failure.[37]

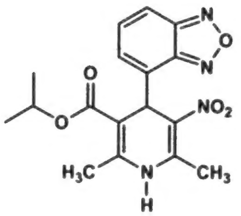
Nimodipine (**8**) possesses a high lipid solubility which enhances its ability to cross the blood brain barrier. This physicochemical property accounts for its potent and beneficial cerebrovascular effects, which include a reduction in cerebral spasm[38] and the treatment of migraine.[39-41]

Isradipine (**9**), and felodipine (**10**) decrease blood pressure by reducing total peripheral resistance, but unlike other CCants that exert a negative inotropic effect, both possess potent vasodilatory properties at doses much lower than those associated with their negative inotropic effects.[42, 43] Structural modification of nifedipine also gave rise to related compounds having effects completely opposite to the traditional CCants. Compounds which enhanced cardiac contractility, as well as the contraction of vascular smooth muscle are known. The prototype of this class of compounds is BAY K 8644 (**11**) which is a calcium channel agonist (Figure 5).[44-46] It was first obtained as a racemate with calcium channel agonist [(-)-(S)-enantiomer] and antagonist [(+)-(R)-enantiomer] properties.[47,48] The racemic mixture displays agonist activity at low concentrations and antagonist activity at high concentrations.[44,49] Other CCags that exhibit similar properties to BAY K 8644 include PN 202-791 (**12**),[50] CGP 28392 (**13**),[51] YC-170 (**14**)[52] and LC 249933 (**15**).[52,53] However, in spite of their desirable cardiac inotropic effect, all these CCags were not useful clinically due to their contraindicated vasoconstrictive effect on vascular smooth muscle.[20]

In 1987, AK-2-38 (**16**, Figure 6) was reported by Knaus *et al.* as having twice the potency of nifedipine on smooth muscle ($IC_{50} = 6.7 \times 10^{-9}$ M vs 1.4×10^{-8} M respectively), and exhibited partial agonist activity on cardiac muscle, a decided advantage as a potential antihypertensive agent.[20] It was the first 1,4-DHP reported to exert a *dual smooth muscle selective CC antagonist and a cardioselective CC partial agonist effect*. The remarkable significance of this statement indicated that future clinical use of calcium agonists to treat congestive heart failure will therefore be dependent upon separating their undesirable vasoconstricting effects from their desirable cardiostimulant properties.[54]


CF₃
O
H₃C
O
NO₂
H₃C
N
CH₃
H

(11)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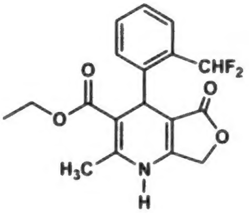
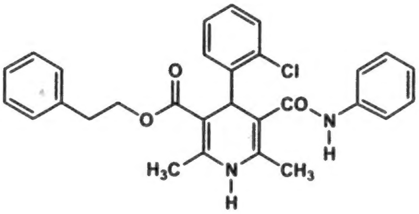
O
O
NO₂
H₃C
N
CH₃
H

(12)


CHF₂
O
O
O
O
H₃C
N
H

(13)


Cl
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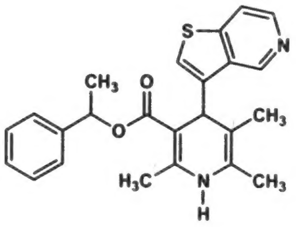
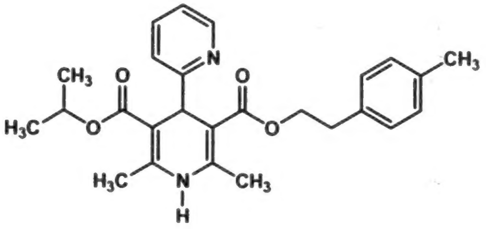
O
CO
N
H
H₃C
N
CH₃
H

(14)


S
N
CH₃
O
O
CH₃
H₃C
N
CH₃
H

(15)

Figure 5: Chemical structures of representative 1,4-DHP CC agonists. BAY K 8644 (**11**), PN 202-791 (**12**), CGP 28392 (**13**), YC-170 (**14**), and LC 249933 (**15**).


N
CH₃
O
O
CH₃
H₃C
O
O
H₃C
N
CH₃
H

(16)

Figure 6: Chemical structure of the 1,4-DHP CC modulator AK-2-38 (**16**).

1.3.4.1.1. Mechanism of action of 1,4-dihydropyridines

The transmembrane entry of extracellular calcium (Ca^{2+}) through CALCIUM CHANNELS[*] (CC) is an important pathway for the excitation-contraction coupling process in muscle.[55] The overall process of Ca^{2+} intake by muscle cells consists of two distinct inward ion currents. The first one (high rate) is a rapid flow of Na^+ ions into the cell, the second one (lower rate) involves the Ca^{2+} entry. There are two major types of Ca^{2+} entry pathways into muscle cells:

I. Potential-dependent channels (PDC), also called voltage-gated channels (VGC), which are activated by membrane depolarization (electrical or K^+-activated), and can be subdivided into at least six different subtypes (Table 1).[56-59]

II. Receptor operated channels (ROC) which are associated with membrane receptors and activated by specific agonist-receptor interactions.

Intake of Ca^{2+} triggers muscle contraction by binding and inhibiting TROPONIN[†]. Once the inhibitory effect of troponin is removed, the intracellular proteins actin and myosin can interact to produce the contractile response. In vascular smooth muscle, Ca^{2+} causes constriction by binding to calmodulin, a specific intracellular protein, to form a complex that initiates the process of vascular constriction.

The L-type calcium channel is the best characterized in terms of its physiology and biochemistry. This calcium channel receptor consists of a heteromeric assembly of five proteins: alpha$_1$ (α_1), alpha$_2$ (α_2), beta (β), gamma (γ) and delta (δ), as shown in Figure 7a.

---

[*] CALCIUM CHANNEL: transmembrane protein responsible for Ca^{2+} uptake process in the cell.

[†] TROPONIN: intracellular protein responsible for the natural suppression of the contractile process.

Table 1: Subtypes of potential-dependent CCs. [a]Subtypes; [b]the effect they exert on the cell membrane; [c]sensitivities toward 1,4-dihydropyridines; [d]membrane potential at which they normally operate; [e]tissue localization.

Subtype[a]	Effect[b]	Influenced by[c]	Membrane potential[d]	Localization[e]
L	Long lasting	CCants and CCags	more than 30 mV	Skeletal muscle: α_{1S} Brain (Neuronal soma & proximal dendrites): α_{1D} Cardiac muscle: α_{1C} Neuroendocrine: α_{1D} Retina: α_{1F}
T	Transient	insensitive to DHP	-50 to -70 mV	Widely distributed (SA node in heart)
N	Neuronal	insensitive to DHP	more than 30 mV	Neuronal (presynaptic)
P	-	insensitive to DHP	more than 30 mV	Neuronal (presynaptic) Cerebellum (Purkinje cells) Neuromuscular junctions
Q	-	insensitive to DHP	more than 30 mV	Cerebellar granule cells Hippocampal pyramidal neurons
R	-	insensitive to DHP	more than 30 mV	-

The delta-subunit is disulfide bonded to the alpha$_2$ subunit, while the gamma-subunit may only be found in skeletal muscle. The alpha$_1$, delta and beta-subunits likely play a role in mediating channel expression and kinetics.[60] The alpha$_1$-subunit is the major pore forming protein of the channel, and it is capable of forming a functional calcium channel. It consists of four domains, each containing six putative membrane spanning regions (S1-S6). The S4 region is thought to be the voltage sensor for the channel, while the loop between S5 and S6 forms at least a portion of the channel pore (Figure 7b). This subunit of the L-type Ca^{2+} channel is found to contain high affinity binding sites for CC modulators.[61]

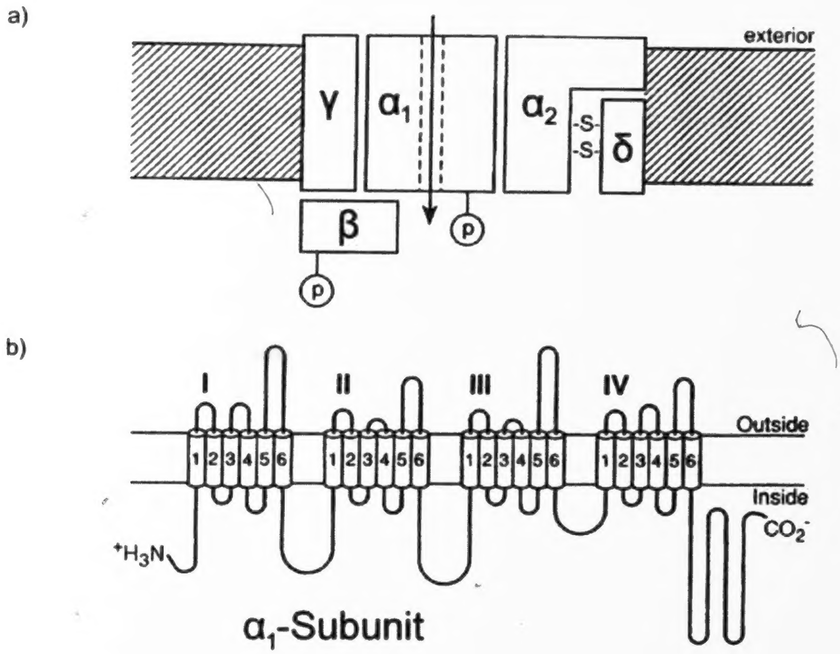


Figure 7: a) L-type calcium channel and its alpha$_1$ (α_1), alpha$_2$ (α_2), beta (β), gamma (γ) and delta (δ) subunits. **b)** The α_1 subunit and spanning regions. Adapted from Triggle, D. J.[62]

1.3.4.1.2. Structure-activity relationships for 1,4-DHP CC modulators

Extensive structure-activity relationships (SARs) have been reported for 1,4-dihydropyridines having C-4 aryl[63-81] and C-4 heteroaryl[19,21-24,82-102] substituents. Changes in the substitution pattern at the C-3, C-4 and C-5 positions alters potency, tissue selectivity and conformation (degree of ring puckering) of the DHP ring, which correlates with CCant activity. The most active compounds exhibit the smallest degree of ring distortion from planarity. There is significant strain in these molecules due to non-bonded interactions involving the ortho-substituents on the C-4 aryl ring, and the C-3 and C-5 ester substituents that is partially relieved by puckering of the DHP ring and distortion of the bond angle about C-4 (Figure 8).[64,103,104]

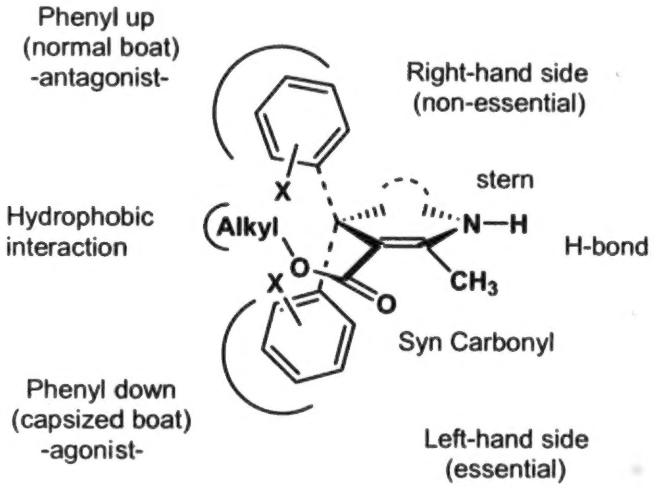

Phenyl up
(normal boat)
-antagonist-
Right-hand side
(non-essential)
stern
X
Hydrophobic
interaction
Alkyl
N—H
H-bond
O
X
CH3
O
Syn Carbonyl
Phenyl down
(capsized boat)
-agonist-
Left-hand side
(essential)

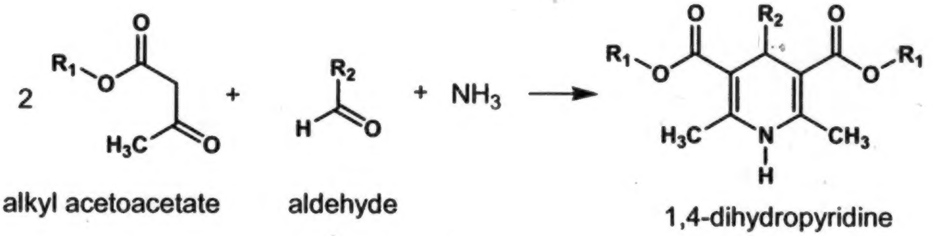
Figure 8: Schematic of essential and non-essential interactions for CC modulators. Left-hand side alkoxycarbonyl interaction for both CCants and CCags. Right-hand side interactions are nonessential. Right and left are defined viewing the DHP ring from N1 (stern) toward C-4 (bow).[105]

There is less data on CCags to make definite conclusions regarding CCag receptor binding interaction.[21,22,96,98,106] Literature data indicates agonist properties are associated with NO_2 or lactone substitution on the DHP ring both of which enhance planarity of the DHP ring and acidity of the NH proton. It has been proposed that the important conformational feature (MOPAC calculations) which differentiates agonist and antagonist activity is the orientation of the C-3 ester (*syn*periplanar for antagonists) or C-3 NO_2 (*anti*periplanar for agonists). In antagonists both ester groups are thought to be oriented in a plane which intersects the plane of the DHP ring with an angle of between 30° and 60°. In the agonist BAY K 8644 (**11**), the 3-nitro group is oriented in the plane of the DHP ring.[107]

Although many CHF treatment options have been identified, additional alternatives are needed to improve further the quality and quantity of life for patients, reduce the number of hospitalizations and decrease health care costs. Therefore, a considerable impetus remains to search for new and more effective cardiotonic drugs, which may result in new treatments for the management of congestive heart failure.[108-110]

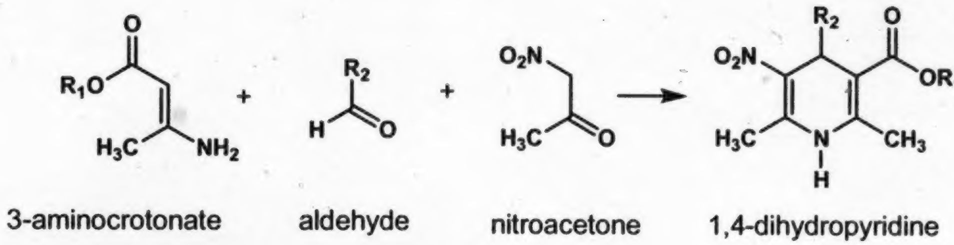
1.3.4.1.3. Synthesis of 1,4-dihydropyridines using Hantzsch and modified Hantzsch reactions.

A number of reviews have been published describing the synthesis, structural elucidation, physical and chemical properties of 1,4-dihydropyridines.[111-114] The original Hantzsch dihydropyridine synthesis involved the reaction of an alkyl acetoacetate with an aldehyde and ammonia, to give the 1,4-dihydropyridine ring (Scheme 1).



Scheme 1: Hantzsch 1,4-dihydropyridine synthesis.

Modification of the classical Hantzsch synthesis enables a variety of 1,4-dihydropyridines to be synthesized. A typical method involves the condensation of an appropriately functionalized 3-aminocrotonate, nitroacetone and an aldehyde (Scheme 2).



Scheme 2: Modified Hantzsch 1,4-dihydropyridine synthesis.

Thermodynamically, 1,4-dihydropyridines are more stable than 1,6- and 1,2-dihydropyridines.[115] Molecular orbital calculations indicate that the lone nitrogen pair of electrones and the electrons of the two olefinic bonds for 1,4-dihydropyridines are delocalized. Thus, electron-withdrawing substituents (such as a nitro group) at the C-3 and/or C-5 positions, that are conjugated with the enamine moiety, enhance stability due to electron delocalization. In contrast, electron-donating groups destabilize the dihydropyridine ring system.[116]

1.4. Rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) is a chronic disorder usually characterized by pain and swelling in the small joints of the feet and hands, although almost any joint in the body may be affected (shoulders, knees, hips, jaws, cervical spine). Persistent joint inflammation often leads to destruction of articular cartilage and bone, as well as permanent deformities. The currently accepted pathogenesis of these disorders involves the combination of unknown antigens with antibodies in the patient's joints, which activates a complement sequence. An antigen-complement-antibody immune complex then precipitates in the synovium and joint fluid, generating the release of chemical mediators that subsequently cause the migration of numerous polymorphonuclear leukocytes phagocytazing the immune complexes. Lysosomal membranes become unstable and discharge hydrolytic enzymes (proteases, collagenases) from the leucocytes and synovial cells. Tissue damage ensues with continuing inflammation, tissue destruction, collagen depolymerization, and loss of physical properties of the connective tissue and joints.[117] Because there is currently no cure for RA, the goal of current therapies is to preserve joint function and limit disease progression by reducing inflammation. The estimated direct and indirect costs of arthritis to Canadian society were calculated to be 5.8 billion (1994, Canadian dollars). The largest portion of indirect costs was wages lost through the inability to work, amounting to 3.7 billion, or 63.4% of the total annual cost of arthritis and rheumatism in Canada.[118]

RA typically begins between the ages of 20 and 50 years, affects 3 times as many women as men, and is associated with substantial morbidity and early mortality. The prevalence of RA in most Western countries, including Canada, is about 1%.[119]

1.5. Nonsteroidal antiinflammatory drugs (NSAIDs)

Inflammation is one of the most important processes involved in the defence of an organism; however, it often progresses to painful or chronically harmful diseases needing pharmacological treatment. The inflammatory response involves many effector mechanisms which produce a multiplicity of vascular and cellular reactions. Many chemical mediators are involved in activating and coordinating the various aspects of the inflammatory process.[120] It is known that vasodilatation, increased microvascular permeability, chemotaxis, cellular activation, pain and finally repair are mediated by the local production and release of several specific mediators. The arachidonic acid derivatives: prostaglandins (PGE_2 in particular), thromboxanes and leukotrienes, together with cytokines, oxygen and possibly nitrogen radicals, play a pivotal role.[121]

The non-steroidal antiinflammatory drugs (NSAIDs), which have been used since the introduction of acetylsalicylic acid (**17**, Figure 9) into medicine in 1899, are an heterogeneous group of compounds which share many pharmacological properties. They represent the main group of drugs used to reduce the untoward consequences of inflammation.[121] NSAIDs are used mainly for sympatomatic relief of mild to moderate pain of headache, arthralgia, myalgia, and neuralgia. Oral NSAIDs are also used for long-term treatment of rheumatoid arthritis, juvenile arthritis, and osteoarthritis. NSAIDs offer only symptomatic treatment for rheumatoid conditions. Although NSAIDs reduce pain, stiffness, swelling, tenderness, they do not revert or arrest the disease process.[117]

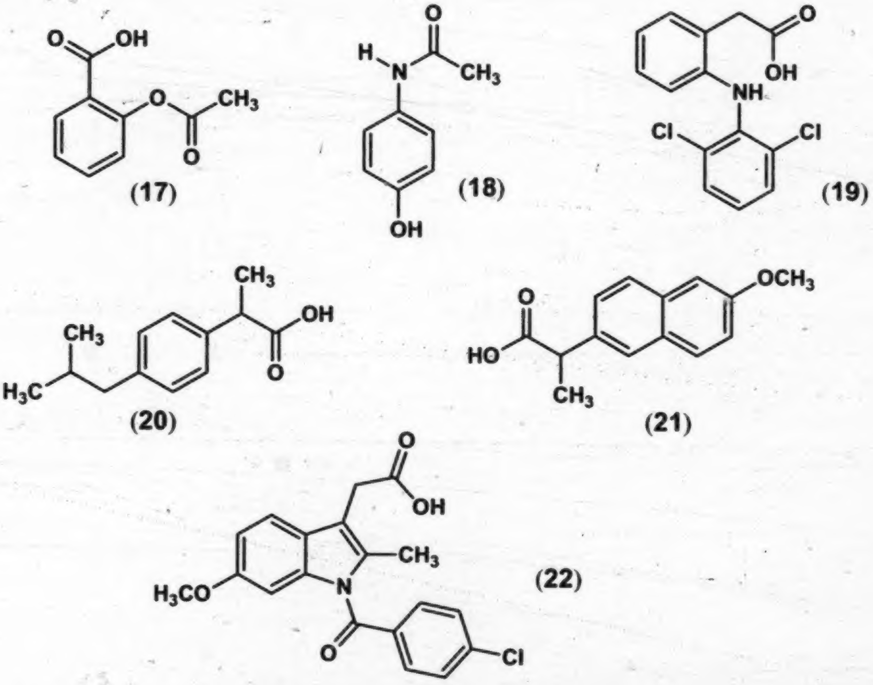


Figure 9: Chemical structures for representative NSAIDs. Acetyl salicylic acid (aspirin, **17**), acetaminophen (**18**), diclofenac (**19**), ibuprofen (**20**), naproxen (**21**), indomethacin (**22**).

The major mechanism of action for NSAIDs is the specific inhibition of the cyclooxygenase (COX) enzymes which catalyze the conversion of arachidonic acid to various eicosanoids including thromboxanes and various prostaglandins (Figure 10).[122-125]

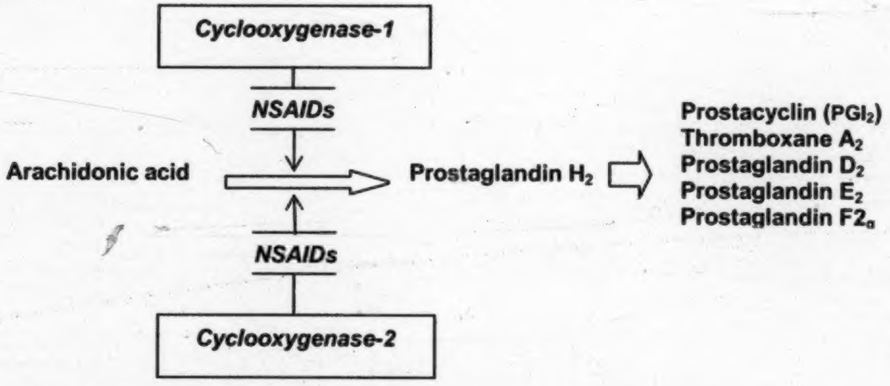

Cyclooxygenase-1
NSAIDs
Arachidonic acid
Prostaglandin H_2
Prostacyclin (PGI_2)
Thromboxane A_2
Prostaglandin D_2
Prostaglandin E_2
Prostaglandin $F2_\alpha$
NSAIDs
Cyclooxygenase-2

Figure 10: Mechanism of action of nonsteroidal antiinflammatory drugs (NSAIDs). Arachidonic acid, liberated from membrane phospholipids in response to multiple stimuli, is converted to prostaglandin H_2 by cytosolic cyclooxygenase-1 and -2. Prostaglandin H_2 is converted by tissue-specific isomerases to multiple prostanoids. Nonsteroidal antiinflammatory drugs (NSAIDs) inhibit both COX-1 and COX-2.

Few other effects are exerted by most NSAIDs on other possible mediators of the inflammatory process.[121] Since prostaglandins, in addition to being important effectors of the inflammatory reactions, also exert specific physiological functions in several organs, inhibition of their synthesis is not devoid of side effects.[126-128] NSAID use is associated with a variety of alterations in gastrointestinal integrity and function.[129,130] Gastric ulcers are one of the most common injuries induced by NSAIDs and are often found in the antrum region of the stomach. These lesions require a long period of time for healing and their potential for perforation and bleeding is considerable.[129] Duodenal ulcers can also be induced by NSAIDs. There is clear evidence that severe complications associated with peptic ulcer disease are often related with recent NSAID consumption.[129,131]

1.6. Pathogenesis of NSAIDs-gastropathy

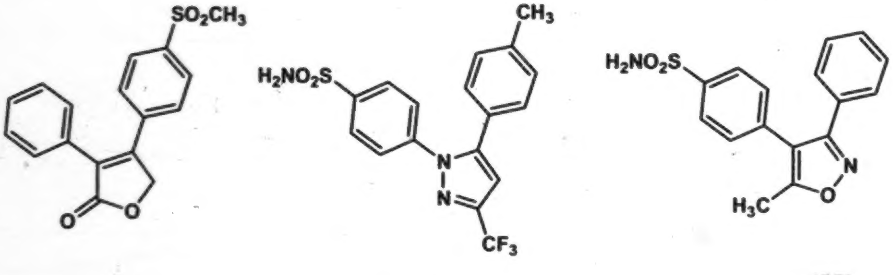
There are two major components to the pathogenesis of NSAID-gastropathy. Some NSAIDs, particularly those that are weak acids, exert topical irritant effects on the epithelium.[132] All NSAIDs have the ability to suppress gastric COX-1-mediated prostaglandin synthesis, and the ability of an NSAID to cause gastric damage correlates well with the ability to suppress gastric prostaglandin synthesis.[133-136] Virtually every component of gastric mucosal defense is mediated, at least to some extent, by prostaglandins: mucus and bicarbonate secretion, mucosal blood flow, epithelial cell replication, and mucosal immunocyte function.[137] Thus, inhibition of prostaglandin synthesis greatly increases the susceptibility of the gastric mucosa to damage induced by acid, pepsin or other luminal irritants. It is the effects of NSAIDs on the microcirculation that are most important in terms of the development of mucosal injury.[132] It has been recognized for many years that NSAIDs reduce gastric mucosal blood flow,[138, 139] which greatly reduces the capacity of the mucosa to cope with back-diffusing acid and to undergo epithelial repair.[140] There is also evidence that damage to the vascular endothelium is a very early event following NSAID administration.[141] This damage appears to be caused by neutrophils.[142] NSAID administration to rats results in an increase in the number of neutrophils adhering to the vascular endothelium in the gastric and mesenteric microcirculation.[135,143,144] This occurs within 30 min of administration of an NSAID, consistent with the period of time required for significant inhibition of prostaglandin synthesis by these drugs.[140] NSAID-induced neutrophil adherence could contribute to the pathogenesis of gastric mucosal injury[145] in two major ways:

I. The factors that trigger the adherence of neutrophils to the vascular endothelium are likely to also trigger the activation of these cells, leading to the liberation of oxygen-derived free radicals and proteases. These substances could mediate much of the endothelial and epithelial injury caused by NSAIDs. Indeed, there is good evidence that reactive oxygen metabolites contribute to the mucosal injury observed following NSAID administration to rats.[146,147]

II. Neutrophil adherence to the vascular endothelium could lead to obstruction of capillaries, resulting in a reduction in gastric mucosal blood flow, and thereby predisposing the mucosa to injury. As mentioned above, a reduction in gastric blood flow following NSAID administration has been reported by many groups,[138,139] and has been shown to occur subsequent to the appearance of 'white thrombi' in the gastric microcirculation.[148]

1.7. Selective cyclooxygenase-2 inhibitors (COX-2 inhibitors)

Nonsteroidal antiinflammatory drugs vary in their relative inhibitory effects on COX-1 and COX-2 enzymes.[125, 149] Aspirin is approximately 166 times more potent an inhibitor of COX-1 as compared with COX-2.[150] In an attempt to overcome the gastrointestinal toxicity and hemorrhagic risk associated with nonselective NSAIDs, selective COX-2 inhibitors were developed for the treatment of inflammation and pain.[151] This class includes rofecoxib (**23**), celecoxib (**24**), and valdecoxib (**25**) (Figure 11).



(23) (24) (25)

Figure 11: Structures of selective COX-2 inhibitors rofecoxib (**23**), celecoxib (**24**) and valdecoxib (**25**).

The relative selectivity toward COX-2 varies; for example, rofecoxib has greater COX-2 selectivity than celecoxib. The recent discovery of two enzymatic isoforms COX-1 and COX-2, encoded by two different genes, generated much hope of developing a more specific and safe approach in the treatment of inflammatory diseases. Initial studies appeared to indicate that COX-1 represented the constitutive isoform responsible for the production of prostaglandins with specific 'housekeeping' physiological functions, as protection of the gastric mucosa and the maintenance of renal perfusion.[149,152-154] On the other hand, COX-2 had a markedly lower expression under basal conditions, but it is the dominant isozyme in inflamed tissues where it was induced by a number of cytokines (interleukin-1, tumor necrosis factor alpha, bacterial toxins) with the apparent exclusive role of producing pro-inflammatory prostaglandins.

The two isozymes possess a similar amino acid sequence (about 60% homology), and similar catalytic sites with the following differences:[155]

COX-1	COX-2	Significance
Ile^{523}	Val^{523}	➔ Changes the conformation of Tyr^{355} residue on the active site, which gives rise to a 2° pocket in COX-2.
His^{513}	Arg^{513}	➔ Plays a key role in *H*-bonding in the COX-2 binding site. ➔ Interaction with the bound drug is a requirement for time-dependent inhibition.
	His^{90}, Gln^{192} and Tyr^{355}	➔ Control the access of ligands to 2° pocket.
$316\ \text{Å}^3$ (active site)	$394\ \text{Å}^3$ (active site)	➔ COX-2 active site (1° and 2°) is 25% larger than COX-1 active site.

Therefore, it seemed reasonable to anticipate that compounds with COX-2 selectivity might possess antiinflammatory and analgesic effects without affecting important physiological processes controlled by prostaglandins formed by the constitutive COX-1 isozyme.[154,156] In particular, these drugs were expected to leave renal function unaffected and to spare the gastric mucosa from erosions, ulcerations and bleedings, which represented the most frequent and troublesome side effect of NSAID therapy. The hypothesis was so attractive that it has led to the synthesis and clinical utilization of a considerable number of selective COX-2 inhibitors, before it was rigorously tested.

This may be the reason behind the recent announcement made by Merck regarding a voluntary worldwide withdrawal of Vioxx® (rofecoxib). A three-year clinical trial revealed a "discernible and confirmed" higher risk of cardiovascular events such as stroke and heart attacks in patients taking the drug to study whether Vioxx® reduces colon polyp recurrence.[157] In another study, the use of rofecoxib in patients with rheumatoid arthritis was associated with a 5-fold higher increase of myocardial infarction compared with patients using the conventional NSAID naproxen.[158]

1.8. NSAIDs in cardiovascular homeostasis

Aspirin is the only NSAID used for prevention and treatment of coronary heart disease. For primary prevention, aspirin has been shown to reduce the risk of cardiovascular events by 15% and myocardial infarction (MI) by 30%.[159] Aspirin has also been shown to reduce the risk of recurrent MI or other thrombotic vascular events by approximately 25%.[160]

Platelets contain predominantly, if not exclusively, the COX-1 enzyme; since platelets are anucleate, there can be no induction of COX-2 enzyme synthesis. Thromboxane A_2 (TxA_2), the major COX-1 product of arachidonic acid metabolism in platelets, causes irreversible platelet aggregation, vasoconstriction, and smooth muscle proliferation.[161,162] In pathologic situations, such as with a ruptured atherosclerotic plaque, platelet aggregation can produce a vascular thrombus. Even at low doses (81 to 325 mg/day)[163] aspirin irreversibly acetylates Ser^{530} of COX-1 isoenzyme, resulting in complete platelet inhibition.[123] This inhibition is substantial (more than 90%), and since anucleate platelets cannot regenerate cyclooxygenase, the effect will be sustained for the life of the platelet.[163] This effect can protect against the development of an occlusive thrombus. However, it also interferes with normal haemostasis and can clinically manifest itself as excessive bleeding.[164] Other nonselective NSAIDs such as naproxen or ibuprofen, cause varying degrees of inhibition of COX-1 and COX-2 (from 50-95%) in a reversible time-dependent fashion. [123,125,165]

PGI_2 is a major product of the macrovascular endothelium and is a potent inhibitor of platelet aggregation, a vasodilator, and an inhibitor of smooth muscle cell proliferation.[165,166] Thus PGI_2 is a physiologic counter-regulatory influence to platelet-derived thromboxane A_2. The relation between the platelet and the vascular endothelium is intricate and represents a balance between inhibiting platelet aggregation in healthy tissue, and facilitating aggregation after vessel injury. Both prostacyclin (PGI_2), produced by the endothelium, and thromboxane (TxA_2), produced by the platelets, are among the factors that participate in maintaining this balance.

On the basis of the observations described above, it has been hypothesized that nonselective NSAIDs and COX-2 inhibitors may differentially alter the balance between platelet aggregation and the endothelial-mediated inhibition of aggregation. The relative reduction in the synthesis of antiaggregatory PGI_2 in the absence of platelet thromboxane A_2 inhibition has been proposed as potentially increasing the propensity to thrombosis *in vivo*.[161,167]

The clinical impact of alterations in cyclooxygenase-mediated, platelet-endothelial interactions is further complicated by the fact that the endothelium also produces other potent antiplatelet factors that do not depend on cyclooxygenase, the most well-known of which is nitric oxide.[151]

1.9. Nitric oxide: the bioregulator

1.9.1. What is nitric oxide?

Nitric oxide (•NO) is one of the smallest molecules found in nature, consisting simply of one atom of nitrogen and one atom of oxygen. It has been known to chemists for more than two centuries. As recently as 1987, this diatomic free radical was widely considered to be just a toxic gas, a pollutant, one of the constituents of acid rain, smog, and tobacco smoke or a product of electrochemical storms. However, discoveries made by 1988, offered overwhelming evidence that •NO was an integral part of normal physiological function, giving way to a major revolution in biomedical research.[168] •NO is synthesized by mammalian cells and functions as a protective, regulatory, and signalling agent. Nonetheless, the fact that •NO acts both as a physiological messenger and as a cytotoxic agent have had a dramatic effect on biological research in the fields of health and disease. Attempts to reconcile such opposing effects have stimulated research into how the internal chemical environment of the cell can modify the actions of a chemical messenger.[169] These crucial bioregulatory and toxic functions, and the awareness that our bodies produce from one to nine mmol of •NO every day,[170] have made this compound one of the most studied molecules in biomedical science. Back in 1992 the editors of the *Science* Journal called •NO "the molecule of the year" and the 1998 Nobel Prize in Physiology or Medicine was awarded to three American scientists, Robert Furchgott, Louis Ignarro, and Ferid Murad, for their discoveries of the roles of •NO as a signalling molecule.[171]

•NO participates in the regulation of the blood-vessel tone, inhibits platelet aggregation and platelet adhesion on the blood vessel walls, and operates in the central and peripheral nervous systems by regulating respiratory functions, the gastrointestinal tract, and the urogenital system. Hence, •NO exhibits a fairly broad range of biological activities.[172]

1.9.2. Physical and chemical properties of nitric oxide

•NO is a free radical, the simplest known thermally stable paramagnetic molecule, with a solubility in water of about 2mM at room temperature and 1mM at 37 °C. The boiling point of this compound is -151.8 °C, while its freezing point is -163.6 °C. In the gas phase •NO is colorless, whereas the liquid and the solid states are blue. This free radical contains 11 valence electrons and has a bond length of 1.15 Å. The eleventh electron is placed on a π^* orbital and it is responsible for •NO's rich chemistry, inducing direct and indirect biological effects. Although •NO is a relatively stable chemical entity in the pure state, it is nonetheless very reactive in biological systems.[168] In aqueous media the oxidation of •NO by O_2 produces nitrite (NO_2^-) and nitrate (NO_3^-):

(1) $\bullet NO + 0.5\ O_2 \rightarrow NO_2$
(2) $2\ NO_2 \rightarrow N_2O_4$
(3) $\bullet NO + NO_2 \rightarrow N_2O_3$
(4) $N_2O_3 + H_2O \rightarrow 2\ NO_2^- + 2H^+$
(5) $N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+$

Reduction of •NO gives NO^-, which rapidly protonates in water and dimerizes, then loses water to form N_2O. Superoxide anion radical (O_2^-) and •NO couple to form peroxynitrite ($ONOO^-$) which upon protonation to ONOOH rapidly rearranges to nitrate and hydrogen ions.[173] •NO forms complexes with transition metals and binds readily to heme-containing proteins, leading to the formation of metal nitrosyl species that can in turn mediate important physiological functions.[174]

1.9.3. The nitric oxide synthase family

Mammalian cells generate •NO from the amino acid L-arginine and use this gaseous mediator to transmit signals between cells or between one part of the cell to another.[175] The conversion of L-arginine to •NO is catalyzed by a special group of heme-containing enzymes called nitric oxide synthases (NOS). The •NO generated is soluble in both water and lipid and diffuses freely within and between cells. It has a half-life of only a few seconds and is rapidly inactivated upon contact with haemoglobin.[175] There are three isoforms of nitric oxide synthases with a range in molecular weight from about 130 to 160 kDa:

I. *Endothelial* (*eNOS*)
II. *Neuronal* (*nNOS*)
III. *Inducible* (*iNOS*)

Two of the isoforms, *n*NOS and *e*NOS, are constitutive enzymes that are regulated by Ca^{2+} influxes. The third isoform, *i*NOS is produced in many cell types, with especially high levels being formed as a response to inflammatory stimuli.[168] The biosynthesis of •NO occurs as L-arginine is oxidized to N^G-hydroxy-L-arginine followed by further oxidation to L-citrulline,[176] as shown in Figure 12.

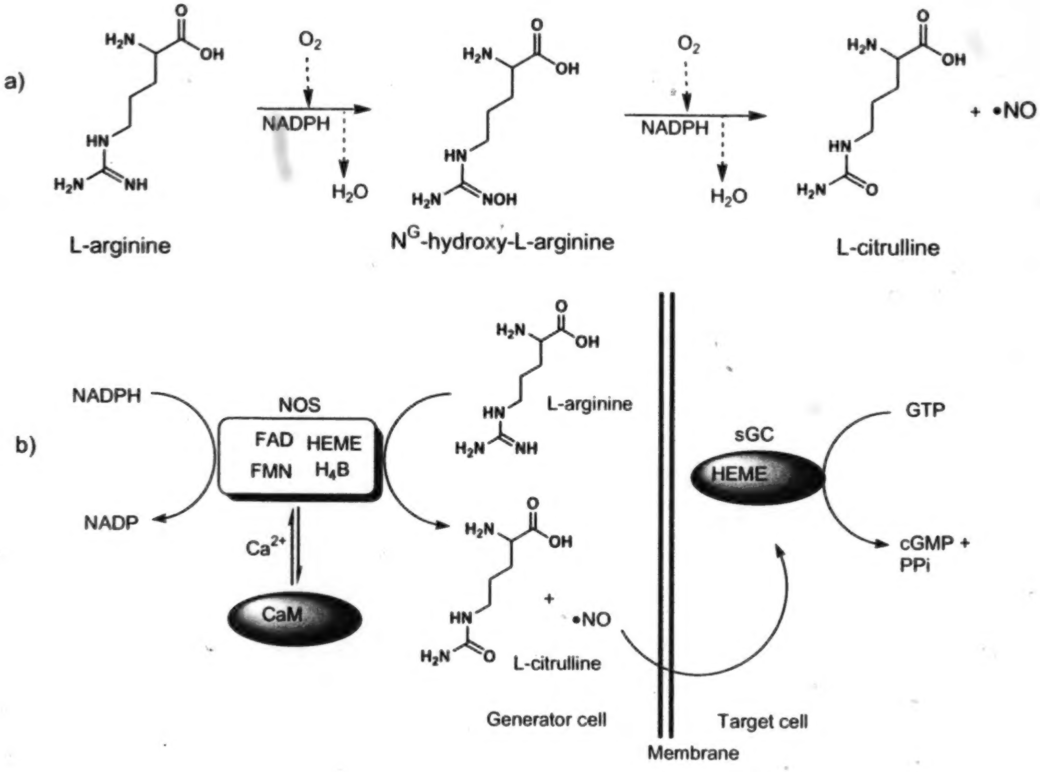

a)
O_2
NADPH
H_2O
O_2
NADPH
H_2O
+ •NO
L-arginine
N^G-hydroxy-L-arginine
L-citrulline
b)
NADPH
NADP
NOS
FAD HEME
FMN H_4B
Ca^{2+}
CaM
L-arginine
+
•NO
L-citrulline
Generator cell
Membrane
Target cell
sGC
HEME
GTP
cGMP +
PPi

Figure 12: a) Biosynthesis of nitric oxide from L-arginine, and b) further diffusion to target cells.

1.9.4. Endothelial nitric oxide synthase (*e*NOS)

The endothelium-dependent relaxation induced by acetyl choline is mediated by the release of •NO from the endothelium, has been detected in arteries, arterioles, veins and venules from a wide range of species, including humans, *in vitro* and *in vivo*.[177,178] Pharmacological inhibition of *e*NOS with substrate analogues such as N^G-monomethyl-L-arginine (L-NMMA) not only impairs the response to acetyl choline and other endothelium-dependent dilators but also causes a substantial increase in the basal tone in resistance vessels.[179] Systemic administration of *e*NOS inhibitors increases arterial blood pressure in animals and healthy volunteers[180] and the hypertension is due to an increase in systemic vascular resistance. Genetically engineered mice that lack the gene for *e*NOS are also hypertensive.[181]

•NO prevents the adhesion of platelets and white cells to endothelium, inhibits the aggregation of platelets and induces dissociation of aggregating platelets.[182] In addition, at high concentrations it inhibits vascular smooth muscle cell growth and may induce apoptosis (programmed cell death).[183] Importantly, these actions of •NO are thought to contribute to "vascular protection", which means the inhibition of atherogenesis and prevention of vessel occlusion.[179] Virtually every cardiovascular risk factor (hypertension, diabetes, hyperlipidemia, smoking) seems to be associated with some sort of reduction in basal or stimulated •NO-mediated dilatation.[184] A relationship between •NO and atherogenesis in humans is also supported by the finding that certain polymorphisms of the *e*NOS gene are associated with increased risk of severe coronary artery disease.[185] The mechanisms of reduced •NO production in cardiovascular disease are not clear but possibilities include changes in essential co-factors for *e*NOS (tetrahydrobiopterin[186]) and the presence of endogenous inhibitors of NOS.[179] It is recognized that increased destruction of •NO through interaction with superoxide anion (O_2^-)[187] may underlie some of the apparent defects in endothelial function seen in disease states. In diabetes, it seems likely that •NO production is normal but a physiological deficit occurs because it is destroyed before it reaches its targets.[188, 189]

1.9.5. Neuronal nitric oxide synthase (*n*NOS)

It is located in central and peripheral neurones and acts as a non-classical neurotransmitter or neuromodulator.[190] *n*NOS has been located in most regions of the brain and it is particularly abundant in the cerebellum, hippocampus and olfactory lobe. •NO participates in conventional neurotransmission and acts as a messenger, sending signals back from the target neuron to the firing axon. •NO has been implicated in excitatory neurotransmission following stimulation of the NMDA glutamate receptor,[191] as a mediator of selective neuronal cell death during development and as a mediator of long-term depression and long-term potentiation (the mechanism by which neurones "remember" previous signals and modify their threshold for activity accordingly).[192] This later process is thought to be one of the fundamental mechanisms underlying memory formation.[179] Pharmacological inhibition of *n*NOS in the central nervous system (CNS) protects neurones from damage caused by excess stimulation of the NMDA receptor, has anti-epileptic effects, alters feeding behaviour and appears to affect memory.[193] Protective effects of *n*NOS inhibitors have been observed in animal models of chemically induced Parkinson's disease, including primates.[194] Consistent with the concept that overproduction of •NO in the CNS may damage neurones, mice lacking the gene for *n*NOS (*n*NOS knockout mice) have reduced damage to experimental stroke.[195]

A network of nitrergic nerves supplies and relaxes smooth muscle throughout the body and forms a major part of the non-adrenergic non-cholinergic nervous system.[196] In the gut, the myenteric plexus contains many nitrergic nerves, which appear to contribute to the relaxant phase of peristalsis. Sphincters are particularly densely innervated with nitrergic nerves, which mediate relaxation of the oesophageal, pyloric and anal sphincters as well as the sphincter of Oddi. Certain blood vessels are also innervated by nitrergic nerves, which contribute the control of vascular tone. In the cerebral circulation nitrergic nerves may be important in increasing the blood flow to the active areas of the brain.[179] Erection of the penis is dependent of the release of •NO from nerves supplying the corpus cavernosum.[197] Nitrergic nerves also contribute to neurogenic dilatation of bronchial smooth muscle and smooth muscle throughout genitourinary tract. Pharmacological inhibition of •NO generation causes increased smooth muscle tone or reduced neurogenic vasodilation in virtually every system.[198]

1.9.6. Inducible nitric oxide synthase (*i*NOS)

It is produced in white blood cells (macrophages) upon stimulation with bacterial lipopolysaccharide (endotoxin) or various pro-inflammatory cytokines (interleukin-1, interferon or tumour necrosis factor-α). The large amount of •NO generated has the capacity to affect multiple enzymes and signalling systems.[179] This is an important host-defence mechanism that can lead to death of certain pathogens. One key mechanism seems to be the highly favoured and fast chemical reaction between •NO and O_2^-, which leads to the formation of peroxynitrite ($ONOO^-$), a powerful oxidant which can cause significant cellular damage, either directly or by stimulating the production of other harmful radicals such as hydroxyl radical. Peroxynitrite can lead to nitration of the amino acid tyrosine[199] and it is thought that this leads to reversible or irreversible changes in function of critical proteins. Either •NO or $ONOO^-$ can also damage DNA, impair repair DNA processes and initiate a signal cascade that leads to irreversible and inevitable cell death. It is believed that killing effects occur at high concentrations (μM range) of •NO whereas only low concentrations (nM range) are required for signalling.[179]

Of the three isoforms of NOS, it is the over-activity of *i*NOS that has been most clearly linked to pathophysiology in animal models. For example, induction of *i*NOS in vasculature is a fundamental mechanism of vascular collapse in experimental septic shock.[200,201] In the gut, induction of *i*NOS contributes to changes in models of inflammatory bowel disease.[202] In the immune system, loss of *i*NOS renders animals more susceptible to infections to certain organisms (e.g. Leishmania, plasmodium).[203-205] However, it is this isoform of NOS that differs the most between humans and animals (80% identity with its murine counterpart).[179]

Induction of *i*NOS is thought to be one of the most important pathogenic mechanisms in rheumatoid arthritis where it might contribute to joint destruction, in asthma,[206] and certain skin disorders.[207]

1.9.7. Biochemical targets for nitric oxide

1.9.7.1. Soluble guanylate cyclase

In nanomolar concentrations, •NO exerts its physiological cell signalling actions. It reversibly activates soluble guanylate cyclase (sGC).[208] At higher concentrations, it affects a wide variety of heme-containing and redox sensitive enzymes and ion channels, and has both reversible and irreversible effects. Soluble guanylate cyclase is widespread and found in cells in every organ system. •NO binds to the heme moiety of the enzyme (Figure 13a) and increases its activity by 400-fold, catalyzing the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) (Figure 13b). Elevation of cGMP levels relaxes smooth muscle in blood vessels, the heart, gut, genitourinary tract, airways, and uterus. Increased levels of cGMP also inhibit platelet aggregation and adhesion, and block the adhesion of white cells to the blood vessel wall.[209]

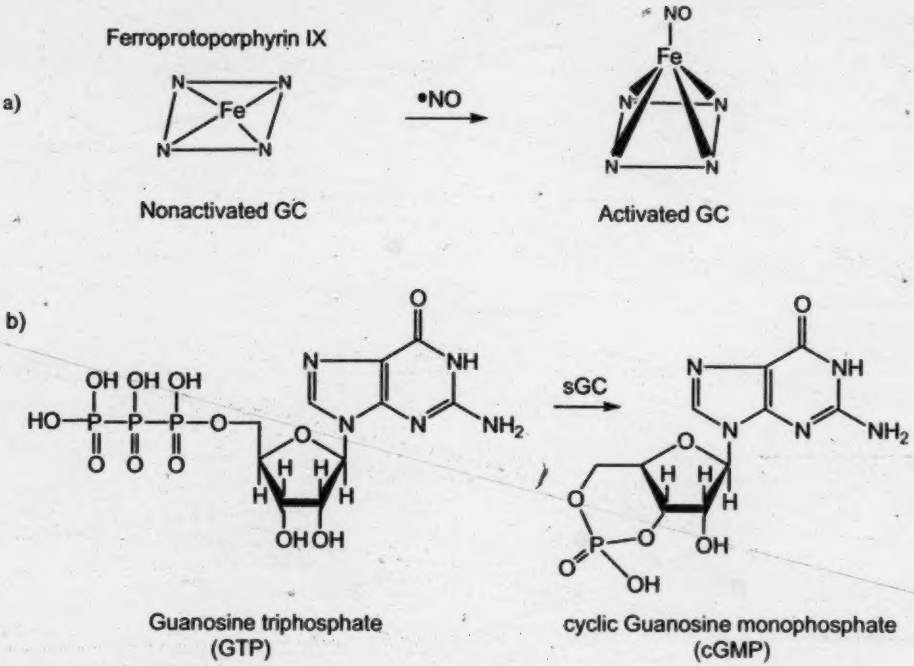

a)
Ferroprotoporphyrin IX
Fe
N
•NO
NO
Nonactivated GC
Activated GC
b)
OH OH OH
HO
P
O
NH
NH2
H
OH OH
sGC
OH
Guanosine triphosphate (GTP)
cyclic Guanosine monophosphate (cGMP)

Figure 13: a) Binding and activation of sGC by nitric oxide, b) catalytic conversion of GTP to cGMP.

1.9.7.2. Mitochondrial respiratory complexes

•NO specifically and reversibly inhibits cytochrome oxidase (complex IV), nitrosothiols inactivate complex I, while peroxynitrite ($ONOO^-$) has multiple effects on different respiratory complexes and can activate the permeability transition pore, which may trigger apoptosis. In physiological concentrations, the effects on complex IV are probably most important and provide a mechanism by which •NO may inhibit or regulate oxygen consumption. It is possible that the local generation of •NO within or close to mitochondria may tonically inhibit oxygen consumption.[210]

1.9.7.3. Nitrosylation of proteins

It has become increasingly clear over the past few years, that •NO can interact with specific activated cysteine residues to alter protein function. A general requirement seems to be that the cysteine is held in an activated state by acidic and basic amino acids that are appropriately aligned in the tertiary structure of the protein.[211] *S*-nitrosylation has been shown to inhibit activity of caspases, ornithine decarboxylase and certain arginine-handling enzymes and can affect ion channel function. Recently it has been shown that the pattern of *S*-nitrosylated proteins differ depending on which isoform of NOS is expressed.[212]

1.9.7.4. Pathological target enzymes

At higher levels (micromolar concentrations) other enzymes become targets for •NO. Heme-containing enzymes with Fe-S clusters including aconitase, NADH dehydrogenase and succinate dehydrogenase, metalloenzymes, ribonucleotide reductase and DNA itself are all susceptible to inhibition or damage.[213]

1.9.8. Nitric oxide donors

When the biosynthesis of •NO is insufficient, serious health problems such as respiratory distress, impotence, unwanted clot formation, and collapsed blood vessels can arise. Molecular •NO has been introduced into wide clinical practice; inhalation of low concentrations of •NO gas (20 ppm) mixed with air produces selective dilation of blood vessels in the lung, and thus it is used in intensive care units to treat disorders such as adult respiratory distress syndrome and persistent pulmonary hypertension of the newborn.[214] However, because •NO's biological half-life is short, continuous release of the gas is necessary, requiring the patient to remain in an inhalation chamber or on a respirator. Moreover, abrupt removal of •NO from the breathing air can cause blood pressure in the lung to rebound to levels higher than those seen before treatment was begun, posing serious limitations to the inhalation therapy.[168]

•NO is so rapidly inactivated by blood, that controlled delivery of this molecule to the organ or cell type where it is needed and without affecting other •NO-sensitive parts of the anatomy remains a challenge. Thus, it is clear that the search for drugs whose transformations in the organism may give rise to •NO is a vigorously progressing line of research.[172] As of today, there are many different types of chemical compounds known to release •NO either *in vitro* or *in vivo* (Figure 14).

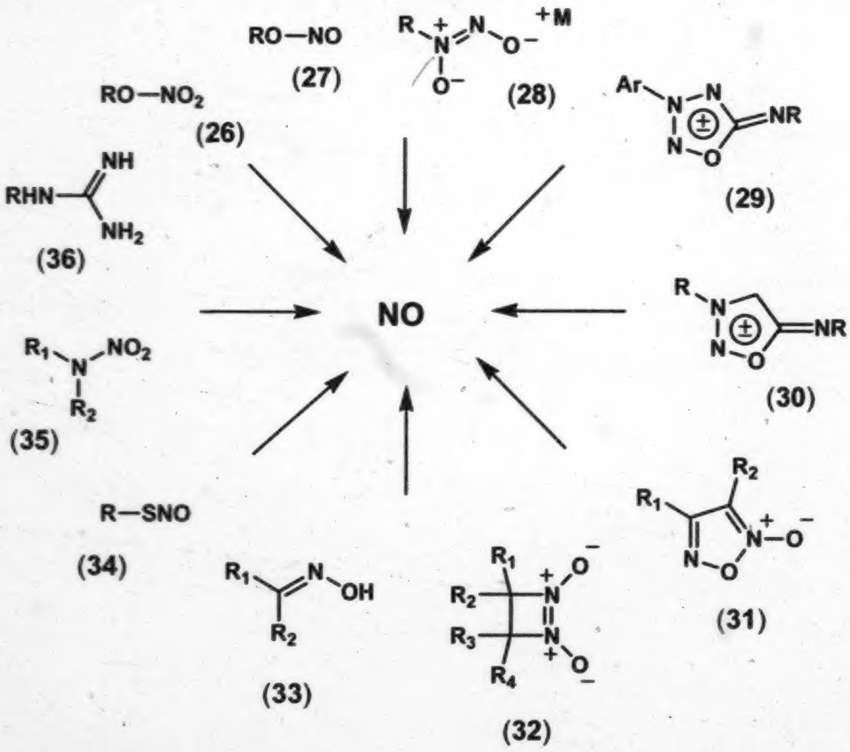


Figure 14: Different •NO-donor functional groups. Organic nitrates (**26** and **27**), diazen-1-ium-1,2-diolates (**28**), mesoionic oxatriazoles (**29**), sydnoneimines (**30**), furoxans (**31**), 1,2-diazete-1,2-dioxides (**32**), oximes

(**33**), *S*-nitroso compounds (**34**), nitramines (**35**), and guanidines (**36**).[172]

Sodium nitroprusside (Figure 15) is a drug used in emergency room situations when the patient's blood pressure is at dangerously high levels. This drug is infused directly into the circulatory system where a one electron-reduction rapidly produces •NO, lowering blood pressure systematically. However, this one electron reduction also leads to the formation of a free cyanide ion, and an iron (II) center that in turn catalyzes the production of highly toxic nitrogen species with consequent toxic effects.[215]

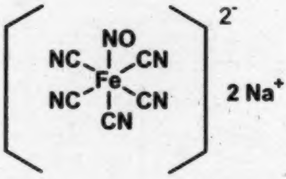


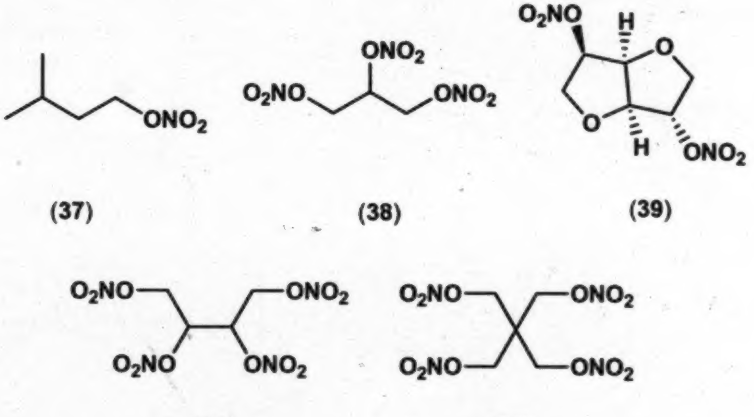
Figure 15: Chemical structure of sodium nitroprusside.

1.9.8.1. Organic nitrates

Organic nitrates have been used for the treatment of acute angina over the last 100 years. Chemically, they were first synthesized by the reaction of simple organic alcohols or polyols with nitric acid.[9] Five members of this class are used clinically today (Figure 16): isoamyl nitrate (**37**), nitroglycerin (**38**), isosorbide dinitrate (**39**), erythrityl tetranitrate (**40**), and pentaerythritol tetranitrate (**41**).

The greater the number of nitro groups, the higher the lipophilicity and the ability to penetrate cell membranes.[172] Production of •NO from organic nitrate esters requires a three-electron reduction, but this metabolism decreases in efficiency on continued use of the drugs, contributing to "nitrate tolerance".[216] Nitrite esters like isoamyl nitrate (**37**) generate •NO by direct reduction or via nitrosation of thiols to form *S*-nitrosothiols that are in turn metabolized to •NO.

Although nitrite esters seem to be a good source of •NO, they are easily hydrolyzed to nitrite ion and are potent nitrosating agents that lead to undesirable, potentially carcinogenic products such as *N*-nitrosamine.[168]



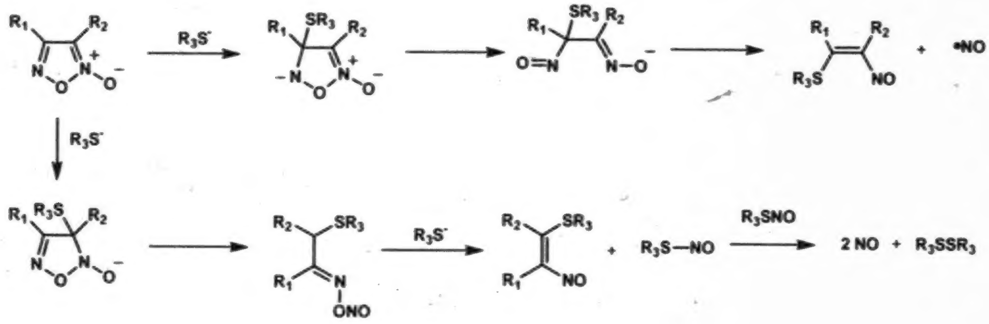
(37) (38) (39)

(40) (41)

Figure 16: Chemical structures of some clinical organic nitrates. Isoamyl nitrate (**37**), glycerol trinitrate (**38**), isosorbide dinitrate (**39**), erythrityl tetranitrate (**40**), and pentaerythritol tetranitrate (**41**).

1.9.8.2. Furoxans

Furoxans represent one class of heterocyclic compounds whose derivatives act as •NO donors which have attracted substantial attention of pharmacologists, biochemists, and chemists. Furoxans are considered prodrugs with a biological activity realized through the sGC-cGMP sequence. The first stage in furoxan degradation is attack by a thiolate anion on positions 3 and/or 4, resulting in ring dearomatization and the possibility of subsequent ring opening followed by the release of •NO (scheme 3).[172]



Scheme 3: Thiol-induced •NO release from furoxans.[171]

Furoxans are thermally stable, acid-resistant compounds that have a low reactivity with electrophiles. Furoxans are less stable towards bases or nucleophilic reagents. Compared to other •NO donors, furoxans possess rather favourable pharmacological properties, since they are slowly transformed and exert a long-term action. Another important distinctive feature of furoxan action is the absence of tolerance.[172]

Several furoxan structures (Figure 17) have been reported to exert biological properties; compound CAS 1609 (**42**) prevents coronary cardiac decompensation when administered orally; CHF 2206 (**43**) inhibits platelet aggregation and eliminates vasospasms caused by noradrenaline four times more effectively than nitroglycerin; compounds **44-49** produce substantial vasorelaxation, among which the benzene derivatives were the most active.[217]

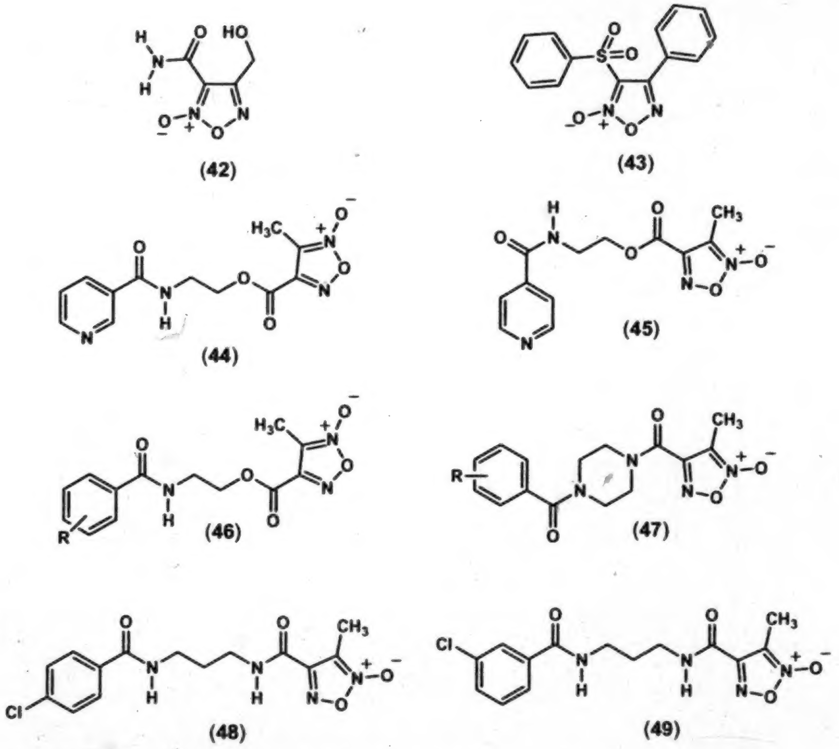


Figure 17: Chemical structures of several biologically active furoxans. CAS 1609 (**42**), CHF 2206 (**43**), and **44-49**.

1.9.8.3. Dizen-1-ium-1,2-diolates

It was not until 1969 that Woodward and Winter first recognized the "methoxazonyl" compounds as distinctive organic functional groups.[218] Early confusion about the nomenclature for these compounds was linked to uncertainties in their structure. Several X-ray crystallographic studies have now offered a more accurate view into the molecular arrangements responsible for their unique chemical structure.[219] As the use of these compounds in biomedical applications increased, Koppenol and Traynham[220] proposed IUPAC names, which were further expanded by Keefer and Hrabie.[219, 221]

Two possible anionic hybrids of resonance (**50** and **51**) proposed for the diazeniumdiolate functional group, are shown in Figure 18.

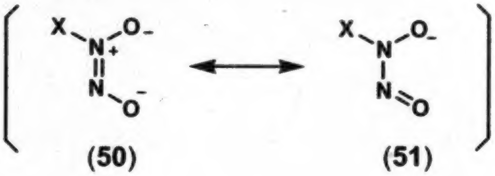
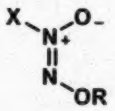
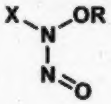


Figure 18: Resonance forms of the diazeniumdiolate functional group attached to an atom "X".

Overwhelming evidence supports structure **50** as the predominant determinant of its physicochemical properties.[222-226] As it is accurately described by Hrabie and Keefer,[227] this is the bonding which gives rise to the group's name. "Diazen" represents the N=N linkage, "ium" the formal positive charge, and "diolate" describes the two negatively charged oxygen atoms. The nitrogen atom attached to substituent X (X = C, N, O, S) is considered position 1, so the full name becomes "diazen-1-ium-1,2-diolate". As shown in Figure 19, a substituent may be present on one of the oxygen atoms, producing either the *O*²-substitued diazeniumdiolate (**52**) or *O*¹-substituted diazeniumdiolate (**53**).



(52)



(53)

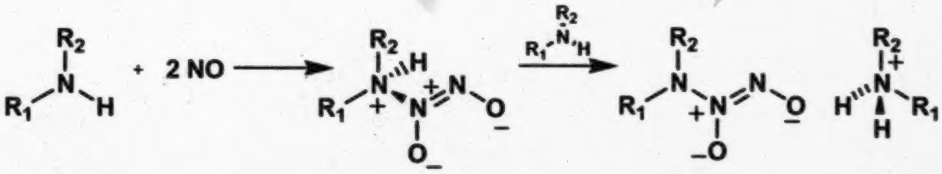
Figure 19: Chemical structures for the two possible *O*-substitutions on diazeniumdiolates. *O*²-substituted diazeniumdiolate (**52**) or *O*¹-substitued diazeniumdiolate (**53**).

N-substituted diazeniumdiolates (X = N) have three main attributes that makes them especially attractive for designing drugs to treat important clinical problems, namely structural diversity, dependable rates of •NO release, and rich derivatization chemistry that facilitates targeting of •NO to specific sites of need. *C*-, *O*-, and *S*-bound diazeniumdiolates are not as attractive as their nitrogen counterparts, since they do not release •NO as the major decomposition product.

The reaction of sulphite ion (SO_3^{2-}) with two equivalents of •NO to give $O_3SN_2O_2^{2-}$ presents an example of a *S*-bound diazeniumdiolate. However, this compound does not revert to •NO when dissolved in water, it produces N_2O and SO_4^{2-}.[228]

1.9.8.3.1. *N*-substituted diazeniumdiolates

As described in section 1.9.2., one of the main chemical features of •NO is its Lewis acidity. It readily accepts electron pairs from nucleophiles or electron donors to form relatively stable adducts. The chemical reaction between primary or secondary amines with •NO (Scheme 4) was first reported by Drago and Paulik in 1960. This study reported solid, relatively stable compounds which, unlike the $O_3SN_2O_2^{2-}$ ion, were able to regenerate both the amine and •NO upon dissolution in water.[228]



Scheme 4: Chemical reaction of secondary amines with nitric oxide.

Since the *N*-diazeniumdiolate functional group is a monobasic acid, in order to keep the adducts produced in a stable anionic form, it is necessary to add a second equivalent of the correspondent amine. Originally, Drago and Paulik carried out these reactions in one of two ways: bubbling •NO into an ether solution of the desired amine at -78 °C at normal atmospheric pressure, or in a closed system in which pressure was applied. Both methods gave the same quaternary ammonium salt of the *N*-diazeniumdiolate; however, the latter provided higher yields. They also observed that ammonium salts were easily converted into more stable sodium salts by adding sodium ethoxide.[228] Even today, direct reaction of •NO with an amine remains the only useful method for their preparation. *N*-diazeniumdiolates obtained from primary amines are far less stable than those synthesized from secondary amines.[229]

It has been found that the rate of •NO release from diazeniumdiolated secondary amines varies greatly with the structure of the substrate. Since the *N*-substituted compounds are quite sensitive to acid, the *O*-protonated *N*-diazeniumdiolates have never been isolated. However, it has been possible to determine the pKa values for some of these compounds because protonation causes a hypsochromic shift in the UV absorption from 250 to 230 nm. Spectrally determined pKa values range from 3.1 for DETA/NO (**54**) to 4.6 for MAHMA/NO (**55**, Figure 20).[230]

Simple monoamines present half-lives that vary from 1.8 s for PROLI/NO (**56**)[231] and 3.0 s for PYRRO/NO (**57**)[232] to 5-6 min for GLO/NO (**58**)[233] as measured at pH 7.4 in phosphate buffer at 37 °C. The polyamine diazeniumdiolates however, exhibit a remarkable degree of variation in the rate of •NO release with relatively minor structural change, with half-lives varying from 1 min for MAHMA/NO (**55**) to 20 h for DETA/NO (**54**).[234]

O^2-unsubstituted diazeniumdiolate half-lives tend to correlate well with their pharmacological durations of action. This suggests that they are minimally affected by metabolism and are essentially different from currently available clinical vasodilators that require redox activation before •NO is released.[235] Kinetic studies investigating the decomposition of *N*-diazeniumdiolates, including determination of the pH dependence of the reaction, revealed that •NO release is initiated by protonation of the amine nitrogen bearing the $N_2O_2^-$

group, following a pseudo-first order rate,[236] but in the case of polyamines, protonation at other nitrogens adds additional complexity at lower pH values.[237]

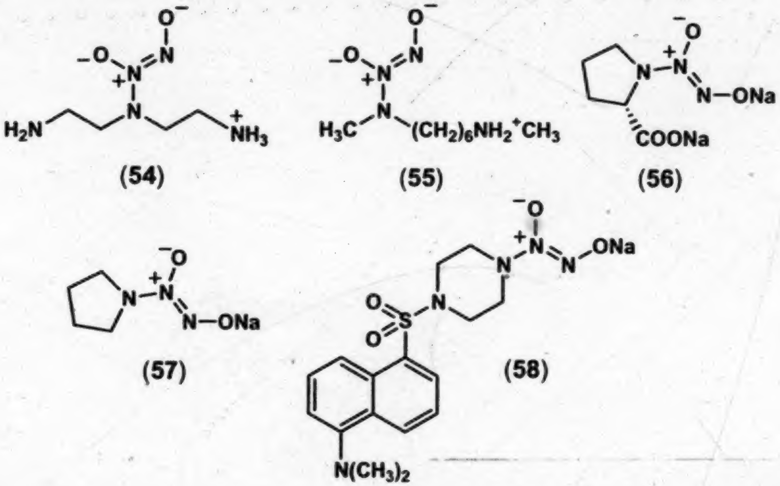


Figure 20: Chemical structures of O^2-unsubstitued *N*-diazeniumdiolates. DETA/NO (**54**), MAHMA/NO (**55**), PROLI/NO (**56**), PYRRO/NO (**57**), and GLO/NO (**58**).

Unlike *C*-diazeniumdiolates, *N*-diazeniumdiolates have not been induced to form stable O^1-derivatives.[227] The simple O^2-alkylated *N*-diazeniumdiolates are generally stable compounds which can be chromatographed, distilled, and/or recrystallized, and many have been prepared with the goal of using the alkyl group as a protecting group that can be removed selectively to regenerate the *N*-diazeniumdiolate, which can subsequently release •NO. O^2-Alkylations can be accomplished using simple alkyl halides[224], epoxides[224], and aryl halides.[238,239] Recently, more highly functionalized alkyl halides[240-242] have been employed. Most O^2-derivatized (as well as underivatized) *N*-diazeniumdiolates are stable to basic reagents and can tolerate many reactions conducted in the presence of base.[227] Some examples of recently reported O^2-alkylated *N*-diazeniumdiolates are shown in Figure 21.

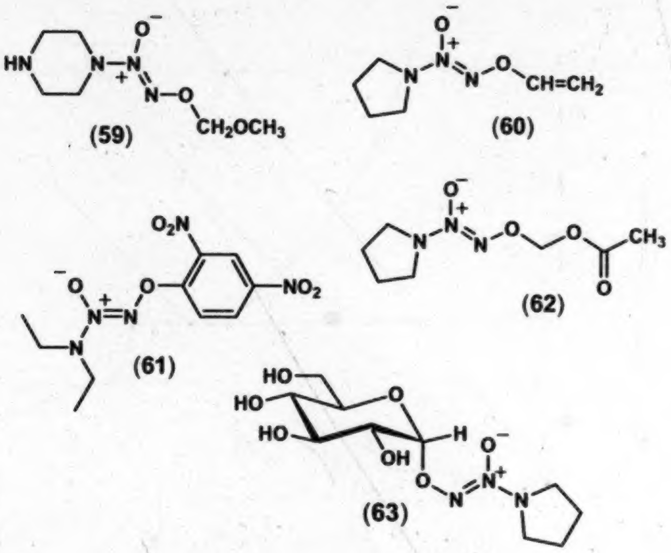

(59) (60) (61) (62) (63)

Figure 21: Chemical structures of some O^2-alkylated *N*-diazeniumdiolates. MOM-PIPERAZI/NO (**59**), V-PYRRO/NO (**60**), O^2-Aryl-DEA/NO (**61**), AcOM-PYRRO/NO (**62**), and β-Glc-PYRRO/NO (**63**).

The acetal MOM-PIPERAZI/NO (**59**) has an extended half-life of •NO release (~17 days at pH 7.4 in phosphate buffer solution) due to the need for the alkyl group to hydrolyze to a mixture of the free diazeniumdiolate, methanol, and formaldehyde before the •NO release can occur.[233]

Novel enzyme selective •NO donors which are activated by hepatocytes in the liver (V-PYRRO/NO, **60**),[232] hydrolyzed by porcine liver esterases (AcOM-PYRRO/NO, **62**),[240] or β-D-glucosidase (β-Glc-PYRRO/NO, **63**),[242] constitute some examples of biologically activated *N*-diazeniumdiolates. The O^2-aryl compound **61** is reported to be activated by nucleophilic aromatic substitution reactions, including the attack from thiolate of the zinc finger portion of an HIV nucleocapsid protein.[239] These compounds act as real prodrugs. V-PYRRO/NO (**60**), for example, was designed and found to be liver selective (activation by cytochrome P450-induced oxidative removal of the vinyl group) in its •NO release via metabolism with enzymes concentrated in that organ. It has been shown to be hepatoprotective in at least three models of fulminant liver failure.[232,243,244]

1.9.9. The •NO-NSAID approach

•NO is now recognized as a critical mediator of gastrointestinal mucosal defence, exerting many of the same actions as prostaglandins in the gastrointestinal tract.[132] Prostaglandins and •NO are both capable of modulating mucosal blood flow, mucus release, and repair of mucosal injury, both mediators inhibit neutrophil adherence and activation. •NO has been shown to reduce the severity of gastric injury in experimental models, just as prostaglandins do.[245,246] As mentioned in section 1.6., reduced mucosal blood flow and adherence of neutrophils to the vascular endothelium appear to be important events in the pathogenesis of NSAID-induced gastric damage. Since •NO has the capacity to block both of these events, Wallace and coworkers[247] have proposed that the linking of an •NO-releasing moiety to an NSAID may reduce the toxicity of the latter (Figure 22).[132]

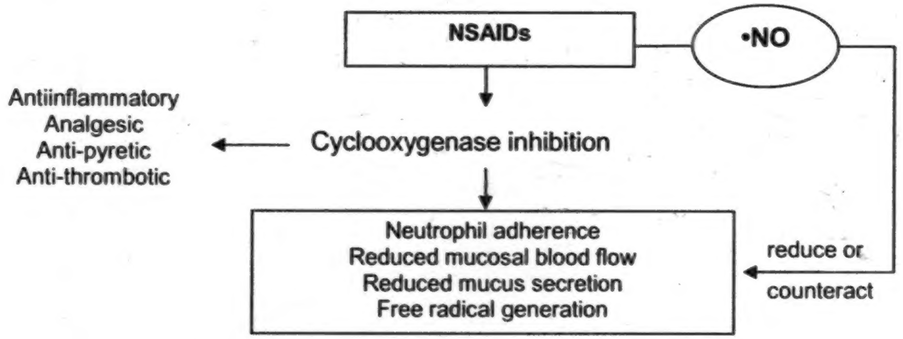

NSAIDs
•NO
Antiinflammatory
Analgesic
Anti-pyretic
Anti-thrombotic
Cyclooxygenase inhibition
Neutrophil adherence
Reduced mucosal blood flow
Reduced mucus secretion
Free radical generation
reduce or
counteract

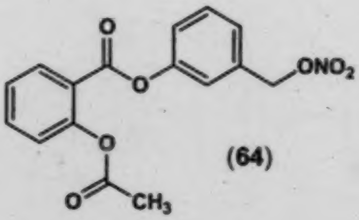
Figure 22: The •NO-NSAID approach.

This hypothesis has now been proven in experimental models of NSAID-induced gastroenteropathy and in early clinical trials. Despite suppressing gastric prostaglandin synthesis, a •NO-diclofenac derivative did not reduce gastric mucosal blood flow.[248] This is presumably attributable to the vasodilator properties of the •NO released from these compounds. It is important to mention, however, that the •NO-NSAIDs do not alter systemic arterial blood pressure in healthy laboratory animals or in healthy human volunteers.[249,250] This observation suggests •NO release must occur at a very slow rate and in amounts too small to significantly affect systemic vascular tone. Pharmacokinetic studies have confirmed the prolonged release of small amounts of •NO from •NO-NSAIDs.[251] While indomethacin induces neutrophil adherence to the vascular endothelium,[252] •NO-NSAIDs do not.[247] Again, it is most likely that •NO released from these agents accounts for the lack of neutrophil adherence. Indeed, •NO-NSAIDs can suppress neutrophil adherence induced by potent chemotaxins, thus expanding the antiinflammatory profile of these drugs relative to the parent drugs.[253]

In animal studies, •NO-releasing derivatives of a wide range of NSAIDs, including aspirin, flurbiprofen, ketoprofen, naproxen and diclofenac, have been shown to spare the gastrointestinal tract, even though they suppressed prostaglandin synthesis as effectively as the parent drug.[247,248,254-256]

When conventional NSAIDs or selective COX-2 inhibitors are administered to rats in which gastric ulcers have been induced mechanically or chemically, a significant delay of ulcer healing is observed.[257,258] In contrast, daily administration of an antiinflammatory dose of an •NO-releasing diclofenac derivative resulted in a significant acceleration of ulcer healing.[259] Likewise, experimental colitis in rats can be exacerbated by treatment with conventional NSAIDs or selective COX-2 inhibitors, such that perforation of colonic ulcers occurs.[260,261] In contrast, •NO-releasing diclofenac was found to be well tolerated by rats with colitis.[254] Rats with arthritis or with cirrhosis also show an increased propensity to develop NSAID-induced damage. In both cases, •NO-NSAIDs were found to be well tolerated.[262] Older rats are known to be more susceptible to NSAID-induced gastric injury.[132] However, studies of •NO-NSAID administration to older rats demonstrated safety in this animal model.[263]

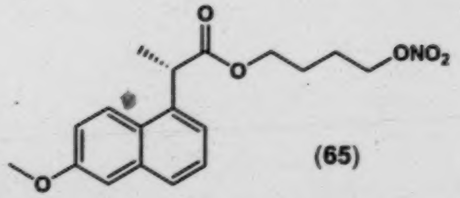
Two recent clinical trials have provided data consistent with the animal studies that showed gastric-sparing effects of •NO-NSAIDs. Of the several •NO-aspirin molecules that have been reported, NCX 4016 (**64**, Figure 23) is perhaps the best characterized, both experimentally and clinically. In one study, twice-daily administration of NCX 4016 for one week did not produce gastric injury in humans, while both of the doses of aspirin that were tested (200 and 450 mg) produced extensive gastric injury.[264] This degree of gastric safety was observed despite the fact that NCX 4016 suppressed whole blood thromboxane synthesis as effectively as did aspirin.



(**64**)

Figure 23: Structure of NCX 4016 (**64**): a •NO-releasing aspirin.

The other recent trial investigated the gastric safety of AZD3582 (**65**, Figure 24), an •NO-releasing derivative of naproxen, the first in a new class of agents called CINODs (COX-inhibiting •NO donors). Twice-daily administration of AZD3582 produced 72% less gastric damage in healthy volunteers than did an equimolar dose of naproxen.[265] This reduction in damage is similar to that observed with COX-2 selective NSAIDs.



(65)

Figure 24: Structure of (2*S*)-4-nitrooxybutyl 2-(6-methoxy-2-naphthyl)propanoate (AZD3582): a •NO-releasing naproxen (**65**).

1.9.9.1. Cardiovascular applications of •NO-NSAIDs

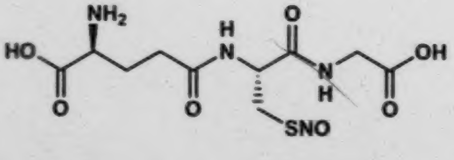
In the latter part of the 20th century, the use of aspirin for the prevention of diseases such as myocardial infarction, stroke and cancer gained wide attention. The evidence for the efficacy of aspirin in long term prophylaxis of myocardial infarction and stroke is convincing,[160,266-269] and such effects are related to its ability to irreversibly inhibit thromboxane A_2 synthesis by platelets, which then reduces platelet aggregation.[270] Low doses of aspirin (10-100 mg/day) used over many days can produce an effective blockade of thromboxane synthesis, with little or no inhibition of prostaglandin synthesis by most tissues.[271, 272] However, even with these low doses of aspirin, there remains a significant risk of bleeding, particularly in the gastrointestinal (GI) tract.[273-275] Furthermore, conventional NSAIDs and selective COX-2 inhibitors can increase blood pressure and exacerbate hypertension, thus interfering with the efficacy of anti-hypertensive medications.[276-278] This creates a problem for patients with hypertension and chronic inflammatory disease, such as rheumatoid arthritis.[279]

As mentioned previously, one of the strategies to develop NSAIDs that do not cause damage to the GI tract, resulted in the introduction and commercial success of COX-2 inhibitors, which induce only about half the incidence of serious GI complications as conventional NSAIDs.[158,280] However, COX-2 inhibitors likely produce some degree of renal toxicity similar to older NSAIDs, and there is evidence to indicate that COX-2 inhibitors increase the risk of thrombosis and myocardial infarction.[158,167,281-283]

The •NO-NSAIDs approach has been shown to exert antiinflammatory and analgesic effects that are at least as potent as those of the parent drug, while sparing the GI tract of injury, and without reducing renal blood flow.[247,248,256,284] •NO-aspirins are of particular interest with respect to prophylaxis of serious cardiovascular disease. •NO-aspirin exerts antithrombotic and antiinflammatory effects beyond those produced by aspirin, but does not cause GI damage.[285-288] In contrast to conventional •NO donors, •NO-aspirins are stable in aqueous solution and release •NO only after enzymatic digestion.[279]

For example, NCX 4016 is well absorbed after oral administration, and it appears that the ester linkage is rapidly cleaved by esterases in the liver and in plasma. In rats, •NO release occurs at low rates over many hours after administration of NCX 4016.[251,289] NCX 4016 does not significantly affect systemic arterial blood pressure in normotensive animals, even when administered intravenously at large doses.[250,285] It is significant that this compound, and other •NO-NSAIDs, can significantly reduce blood pressure in animals with pre-existing hypertension.[289-291]

S-nitrosoglutathione (**66**, Figure 25) is another •NO-donor reported recently. It preferentially inhibits platelet aggregation at doses that barely cause vasodilation, and it appears to be more arterio-selective than conventional nitrovasodilators.[292,293]



(**66**)

Figure 25: Chemical structure of *S*-nitrosoglutathione (**66**).

Atherosclerosis is the most common cause of acute myocardial infarction. It is an inflammatory process, the early stage of which consists of vascular lesions that are characterized by proliferation and activation of monocytes/macrophages, and increased proliferation of vascular smooth muscle.[294] Early lesions in atherogenesis are characterized by vascular endothelial-cell dysfunction, which in turn is characterized by impaired endothelial-derived •NO production and impaired endothelial dependent vasodilation.[269,294,295] Therefore, it has been suggested that the reduced production of •NO might contribute to the increased proliferation of monocytes/macrophages and of vascular smooth muscle that occurs in atherosclerosis.[279]

In experimental animal models, two different •NO-donors (NCX 4016 and *S*-nitrosyl-acetyl-penicillamine) inhibited the proliferation of vascular smooth muscle through a mechanism involving the release of •NO.[296] •NO can inhibit the proliferation of vascular smooth muscle cells via at least two separate pathways:

a) N^G-hydroxyarginine, the principal intermediate in the conversion of arginine to citrulline and •NO by NOS, is a potent competitive inhibitor of arginase.[297-299] Arginase has an essential role in cell growth and wound healing.

b) •NO has been shown to be an inhibitor of ornithine decarboxylase (ODC) acting by a cGMP-independent mechanism.[296,300,301] Ornithine is the substrate from which polyamines, that are essential to mammalian cell proliferation, are derived. Using recombinant ODC, Ignarro and colleagues[296] confirmed that •NO could directly inhibit the activity of ODC by *S*-nitrosylating a cysteine residue in the active site.

This antiproliferative effect might be important in the prevention of RESTENOSIS[*] a common problem associated with percutaneous transluminal coronary ANGIOPLASTY[†] (Figure 26) the most widely used treatment for atherosclerosis.[279] The development of restenosis is an important limitation of this widely used clinical procedure, with re-narrowing of the arterial lumen being caused primarily by HYPERPLASIA.[‡] [302-304] Restenosis has been suggested to occur as a consequence of impaired •NO production by the damaged endothelium.[305] Indeed, administration of the precursor of •NO (L-arginine) has been shown to reduce restenotic lesion formation and monocyte recruitment in rabbits fed cholesterol.[306]

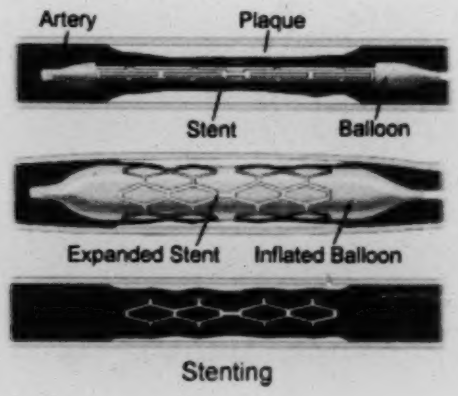


Figure 26: Percutaneous transluminal coronary angioplasty, is often used to open blocked arteries. In this procedure, **A**: a catheter (a thin tube) is inserted into a forearm or groin artery and threaded into the narrowed heart artery; **B**: the catheter has a tiny balloon at its tip that is repeatedly inflated and deflated to open and stretch the artery, improving blood flow; **C**: the tube is removed and often a *stent* (a small metal mesh tube) is inserted to keep an artery open after an angioplasty. The stent stays permanently in the artery. *(Courtesy by: Texas Heart Institute, © 1996 - 2004, http://www.tmc.edu/thi/cad.html).*

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[*] RESTENOSIS: recurrence of a narrowing of a blood vessel after corrective surgery or angioplasty has been performed.

[†] ANGIOPLASTY: an operation for enlarging a narrowed artery by introducing, through the skin, a balloon-tipped catheter into the artery, and dilating the lumen of the artery on withdrawal of the inflated catheter tip.

[‡] HYPERPLASIA: an increase in the number of cells in a tissue, such that the bulk of the tissue is increased.

Aspirin is frequently given to patients who are undergoing angioplasty, but it has only modest therapeutic effects on restenosis, and carries the risk of GI ulceration and bleeding mentioned above. Recently, Napoli *et al.*[307] showed that treatment of hypercholesterolaemic mice that had undergone angioplasty with NCX 4016 resulted in a marked reduction in the degree of restenosis and macrophage deposition at the site of vessel damage. The effects observed with NCX 4016 were considerably greater than those of aspirin and, on a molar basis, lower doses of NCX 4016 were required to produce a beneficial effect. In the same study, the authors also showed that the administration of a •NO donor alone, although producing beneficial effects in the mouse model, did not produce as great an effect as the •NO-releasing aspirin.[307]

Recruitment of white cells (neutrophils and other leukocytes) to a site of wall-vessel damage contributes to the development of a thrombus or atherosclerotic plaque, and to the proliferation of vascular smooth muscle.[279] Unlike aspirin, which is classified as an antiinflammatory drug that can increase leukocyte adherence to the vascular endothelium,[144] NCX 4016 does not cause leukocyte adherence to the vascular endothelium; rather, it can suppress such adherence that is induced by pro-inflammatory mediators. Treatment of rats with NCX 4016 was found to reduce markedly the increase in leukocyte adherence to MESENTERIC VENULES[•] that was stimulated by exposure of the vessels to the chemotactic peptide fMLP.[253] These effects were attributed to the release of •NO from the drug, which like other •NO donors could inhibit leukocyte adherence in a similar manner.[253,308]

[•] MESENTERIC VENULES: small veins in the abdomen that carry blood from the digestive tract to the portal vein, and through that vessel, to the liver.

Using a rabbit model, Rossini *et al.*[309] showed that NCX 4016, but not aspirin, dose-dependently reduced ISCHEMIA-REPERFUSION[*]-induced myocardial damage and dysfunction. These authors reported that the beneficial effects of NCX 4016 appear to be due to the release of •NO from this compound. Thus, •NO could inhibit several cellular events that would otherwise lead to inflammation, coronary microcirculation obstruction, arrhythmias and myocardial tissue necrosis. In the same set of experiments, aspirin and a number of selective COX-2 inhibitors were found to exacerbate myocardial damage and dysfunction induced by ischemia-reperfusion.[310]

During ischemia, COX-2 is upregulated in the myocardium, and the production of PROSTACYCLIN[†] from this enzyme acts to preserve myocardial blood flow.[310-312] This increase in prostacyclin synthesis reduces the vasospasm that occurs as a result of ischemia, partly by reducing the vasoconstrictive effects of endothelin 1, which has been shown to be released into plasma during acute myocardial ischemia.[313] Interestingly, at the highest dose tested, Rossini *et al.*[310] found that NCX 4016 suppressed prostacyclin synthesis (comparable to suppression produced by aspirin and selective COX-2 inhibitors); however, delivery of •NO from NCX 4016 could compensate for the lack of prostacyclin. Although conventional •NO donors can protect the stomach against NSAID-induced gastric damage, they do not do so as effectively as NSAIDs (including aspirin) that are chemically linked to an •NO-releasing moiety.[314] •NO-aspirin can exert antiinflammatory and antithrombotic activities through several mechanisms, which extend beyond COX-dependent and cGMP-dependent mechanisms,[315] since some activities of •NO-aspirin are not reproduced by any concentration of the parent drug.

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[*] ISCHEMIA-REPERFUSION: a local reduction of blood flow, usually due to an obstruction, which is followed by a restoration of blood flow.

[†] PROSTACYCLIN: endogenous chemical produced by various cells, including those in the inner lining of blood vessels, which can cause a widening of blood vessels (thereby increasing the flow of blood) and prevent platelets from sticking to one another.

2.0 Research objectives.

2.1. Nitric oxide-releasing calcium channel modulators.

1,4-Dihydropyridine CCags represent one class of positive inotropes that increase cardiac contractile force without increasing heart rate. However, their adverse CC agonist vasoconstrictive action makes them unsuitable for the treatment of heart failure.[316] We hypothesize that:

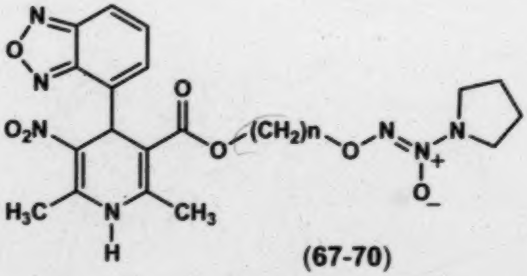
➔ *Tissue selective •NO-CC modulators (1,4-DHP's) that exhibit a dual cardioselective CC agonist effect, in conjunction with a smooth muscle selective CC antagonist vasorelaxant effect, would satisfy the clinical requirements for the treatment of congestive heart failure (CHF), by increasing the force of the heart contractions, while simultaneously inducing a CC vasorelaxant effect, enhanced by the release of •NO directly into peripheral blood vessels.*

Among the major classes of •NO donors, organic nitrates,[26, 102, 317-319] furoxans,[320-322] and nitrosothiols have been investigated as •NO-dihydropyridine drug hybrids. However, nitrosothiol-1,4-DHP hybrids were not considered to be suitable candidates for our research, because nitrosothiols are known to be highly unstable.[323] In contrast, the chemical and pharmacological advantages of *N*-diazeniumdiolates relative to organic nitrates or furoxans, prompted us to investigate the use of a *N*-diazeniumdiolate which is a more attractive •NO donor moiety. In this regard, there are three possible positions in which the *N*-diazeniumdiolate could be attached to CC modulators:

1) via O^1-substitution
2) via O^2-substitution, or
3) via N^1-substitution

Since O^1-alkyl-*N*-diazeniumdiolates are reported to be highly unstable, it was decided to explore the more stable O^2- and N^1- derivatives.

Project 1: It was of interest to acquire structure-activity relationships for 1,4-dihydropyridine CCags possessing a O^2-alkyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate ester moiety as the nitric oxide donor group (Figure 27).

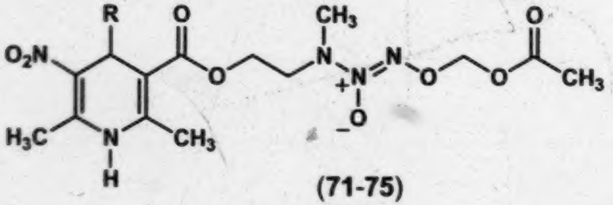


(67-70)

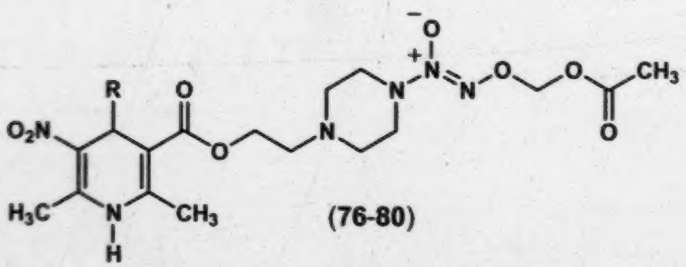
Figure 27: 1,4-dihydropyridine CCags possessing a C-5 substituted O^2-alkyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate; n = 1 (**67**), 2 (**68**), 3 (**69**), and 4 (**70**).

The two parts of these hybrid ester prodrugs, the CC modulator 1,4-dihydro-2,6-dimethyl-4-(benzofurazan-4-yl)-3-nitropyridine-5-carboxylate, and the •NO donor 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate, can be attached by a oligomethylene spacer (n = 1-4). These compounds (**67-70**) are expected to undergo hydrolysis in the liver, or by serum esterases, to release the parent compounds. The percent •NO released will be dependent upon the degree of hydrolysis induced by these enzymes. As reported by Davies,[237] it is also possible that under acidic conditions, protonation of the diazeniumdiolate N^1 initiates •NO release. Based on the fact that hybrid •NO-1,4-DHPs possessing a nitrooxyalkyl C-5 ester substituent showed important CC modulation activities, it was expected that compounds **67-70** could be active CCags even if they are not hydrolyzed by esterases.

Project 2: the objective of this study was to determine structure-activity relationships for 1,4-dihydropyridine CCags having a O^2-acetoxymethyl-1-(*N*-ethyl-*N*-methylamino)diazen-1-ium-1,2-diolate, or O^2-acetoxymethyl-1-(4-ethylpiperazin-1-yl)diazen-1-ium-1,2-diolate, C-5 ester substituent (Figure 28). The diazeniumdiolate moiety is attached to the CCag modulator, via a N^1-ethyl spacer (**71-75**) or via a N^1-(4-ethylpiperazin-1-yl) spacer (**76-80**).



(71-75)



(76-80)

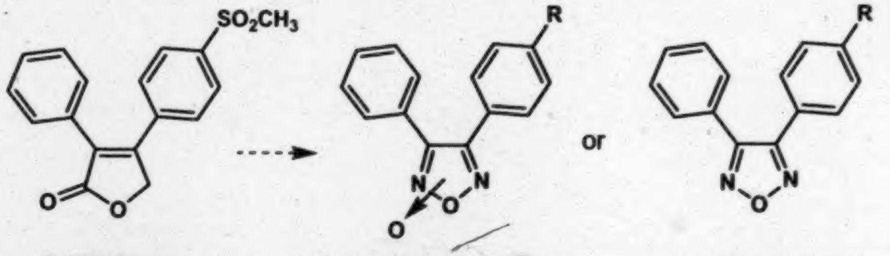
Figure 28: 1,4-Dihydropyridine CCags possessing a O^2-acetoxymethyl-1-(*N*-ethyl-*N*-methylamino)diazen-1-ium-1,2-diolate (**71-75**), or O^2-acetoxymethyl-1-(4-ethylpiperazin-1-yl)diazen-1-ium-1,2-diolate (**76-80**) C-5 ester substituent. R = benzofurazan-4-yl, 2-pyridyl, 3-pyridyl, 4-pyridyl or 2-(trifluoromethyl)phenyl.

O^2-unsubstituted *N*-diazeniumdiolates derived from lower alkyl secondary amines are unstable, resulting in fast •NO release in water ($t_{1/2}$ = 1 second to 5 minute range).[231-233] Therefore, it was necessary to protect the O^2-position with an acetoxymethyl group to increase stability and facilitate handling of samples throughout the chemical and *in vitro* evaluation process.

2.2. Nitric oxide-releasing non-steroidal antiinflammatory drugs.

Drugs classified as •NO-NSAIDs suppress COX-2 derived prostaglandin synthesis as effectively as the parent drugs, and they have been found to exert comparable antiinflammatory and antipyretic activity to the parent NSAID drug. In this regard, •NO-NSAIDs constitute one of the more promising approaches for the design of drugs which are devoid of the potential adverse cardiovascular effects associated with use of selective COX-2 inhibitors, and which elicit a decreased ulcerogenicity relative to that frequently observed on long-term use of traditional NSAIDs. However, the majority of •NO-NSAIDs currently under clinical trials are classified as organic nitrates, which require a 3-electron metabolic reduction to produce •NO. The efficacy of this reduction decreases upon long-term use due to escalating nitrate tolerance.[324] 1,2,5-Oxadiazole-2-oxides (furoxans) represent one class of heterocyclic compounds, that are thiol-dependent •NO donor agents, which possess rather favorable pharmacological properties such as slow •NO release. Unlike nitrates, furoxans do not produce tolerance. It was therefore hypothesized that:

➔ *Replacement of the 2-(5H)furanone central ring present in rofecoxib by an isosteric 1,2,5-oxadiazole-2-oxide ring would maintain COX-2 inhibitory activity. Furthermore, the simultaneous thiol-assisted •NO release from furoxan ring would counteract potential adverse cardiovascular effects associated with the use of selective COX-2 inhibitors.*

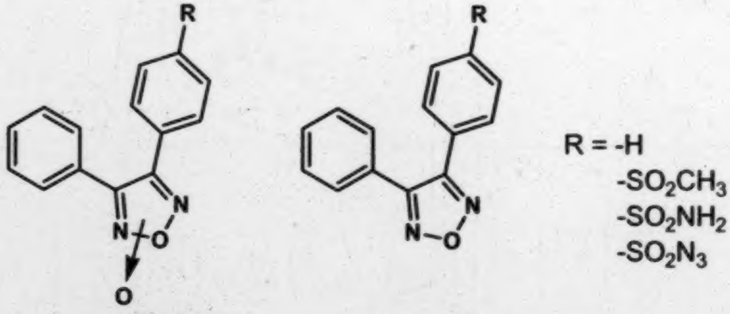


2-(5H)furanone (rofecoxib)

1,2,5-oxadiazole-2-oxide central ring

1,2,5-oxadiazole central ring

Project 3: It was of interest to acquire structure-activity relationships for 3,4-diphenyl-1,2,5-oxadiazole-2-oxides, and the corresponding *N*-deoxy 3,4-diphenyl-1,2,5-oxadiazoles analogs (Figure 29), as hybrid cyclooxygenase (COX) inhibitor / •NO donor agents.



(81-84) **(85-88)**

R = -H
$-SO_2CH_3$
$-SO_2NH_2$
$-SO_2N_3$

Figure 29: 3,4-diphenylfuroxans (**81-84**) and 3,4-diphenylfurazans (**85-88**) possessing a hydrogen (**81**, **85**), methylsulfonyl (**82**, **86**), aminosulfonyl (**83**, **87**), or azidosulfonyl (**84**, **88**) substituent.

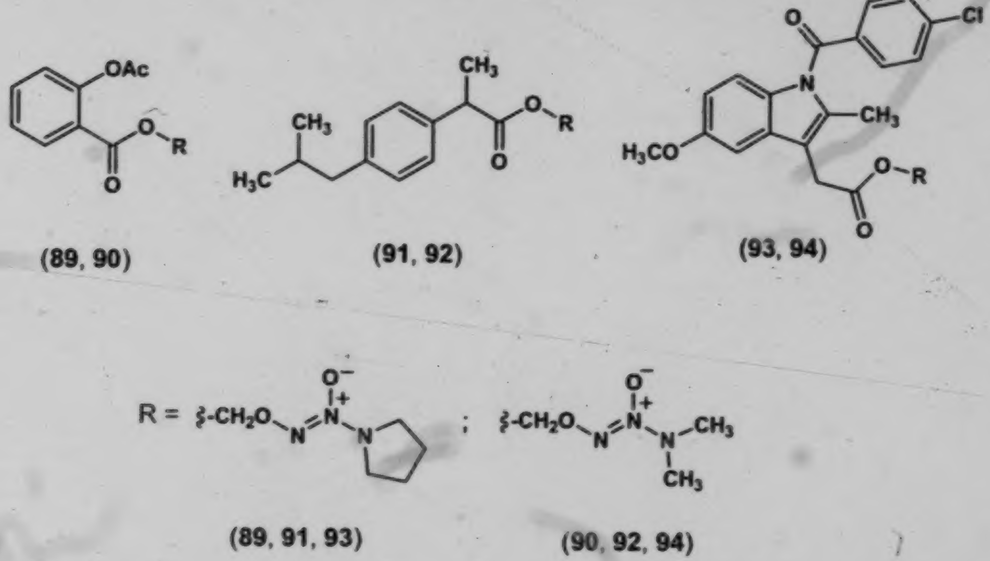
The 3,4-diphenylfuroxans (**81-84**) and 3,4-diphenylfurazans (**85-88**) both possess COX-2 pharmacophores such as the methylsulfonyl ($-SO_2CH_3$) present in rofecoxib, aminosulfonyl ($-SO_2NH_2$) present in celecoxib, and the azidosulfonyl ($-SO_2N_3$) group that was recently reported by the Knaus research group to insert deep inside the 2°-pocket of the COX-2 enzyme.[155]

In the case of compounds **81-84**, the relative position of the oxygen atom on the heterocyclic central ring determines whether the pharmacophore moiety is attached to the C-3 or C-4 phenyl ring, thereby giving rise to two regioisomers.

The chronic use of traditional NSAIDs is frequently associated with alterations in gastrointestinal integrity and function[129,130] which results in the development of gastric ulcers.[325] In this regard, the gastric irritant effect of aspirin (**17**) can be a deterrent to its long-term use for the prophylactic prevention of adverse cardiovascular events such as stroke and myocardial infarction.[326] The •NO-NSAID approach has already been employed with relatively good success in the design, synthesis and biological evaluation of gastrosparing antiinflammatory drugs. However, like the •NO-CC modulators, all •NO-NSAIDs undergoing clinical trials possess an organic nitrate as the •NO donor group. The beneficial chemical and pharmacological advantages of *N*-diazeniumdiolates, prompts us to hypothesize that:

➔ *Hybrid •NO-NSAID ester prodrugs possessing a 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate or 1-(N,N-dimethylamino)diazen-1-ium-1,2-diolate moiety, would be expected to improve the •NO release profile compared to that observed for organic nitrates which require a more metabolically demanding three-electron reduction for the release of •NO, or a thiol cofactor such as L-cysteine or glutathione required for the release of •NO from furoxans.*

Project 4: It was therefore of interest to acquire structure-activity relationships and assess ulcerogenic properties of nitric oxide-releasing non-steroidal antiinflammatory drugs (•NO-NSAIDs), derived from representative analgesic and antiinflammatory drugs such as aspirin, ibuprofen and indomethacin, as hybrid cyclooxygenase (COX) inhibitor / nitric oxide donor agents (Figure 30).



(89, 90)

(91, 92)

(93, 94)

(89, 91, 93)

(90, 92, 94)

Figure 30: nitric oxide-releasing non-steroidal antiinflammatory drugs (•NO-NSAIDs) possessing a 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (**89**, **91**, **93**) or 1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate (**90**, **92**, **94**) ester prodrug moiety.

The NSAID (aspirin, ibuprofen or indomethacin) and the •NO-releasing moieties [1-(pyrrolidin-yl)diazen-1-ium-1,2-diolate or 1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate] were attached via a one carbon methylene spacer, which is expected to undergo metabolic activation (hydrolysis) by non-specific esterases. The extent of *in vitro* •NO release in the presence of liver or serum esterases would provide an indirect measurement of the extent of ester cleavage and then elimination of a molecule of formaldehyde which must occur prior to •NO release from the diazen-1-ium-1,2-diolate moiety.

3.0. Materials and Methods.

3.1. General.

Melting points were recorded with a Thomas-Hoover capillary apparatus and are uncorrected. ^{1}H NMR spectra were acquired using a Bruker AM-300 spectrometer (300 MHz). Infrared spectra were recorded using a Nicolet IR-500 Series II spectrometer. Ultraviolet (UV) spectra and quantitative analyses were measured using a Philips PU 8740 UV/VIS scanning spectrophotometer. Silica gel column chromatography was carried out using Merck 7734 (60-200 mesh) silica gel. Microanalyses were acquired using a CHNS-O EA-1108 Elemental Analyzer (CarloErba Instruments) and the values were within ± 0.4% of theoretical values for all elements listed. X-ray analysis was recorded on a Bruker PLATFORM/SMART 1000 CCD diffractometer. Crystallographic data (excluding structure factors) have been deposited at the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 252139. Acetyl salicylic acid (**17**), racemic ibuprofen (**20**) and indomethacin (**22**) were purchased from the Sigma Chemical Co. O^2-sodium 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (**57**),[232] O^2-(chloromethyl)-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (**95a**),[241] the 1,4-dihydro-2,6-dimethyl-3-nitro-4-[2-pyridyl, 3-pyridyl, 4-pyridyl, 2-(trifluoromethyl)phenyl]pyridine-5-carboxylates (**121**-**124**),[321] chloromethyl acetate,[327] and 1-[4-(methylsulfonyl)phenyl]-2-phenylethene (**126**)[328] were prepared according to reported literature procedures. Nitric oxide gas was purchased from BOC Scientific (Burlington, ON). All other chemicals were purchased from the Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. The *in vivo* calcium channel modulation, antiinflammatory and ulcer index assays were carried out using protocols approved by the Health Sciences Animal Welfare Committee at the University of Alberta. Molecular modeling experiments were performed on a Sillicon Graphics Workstation (Octane 2) equipped with a R14000A microprocessor using the Insight II software version 2000.1 (Accelerys Inc.).

3.2. Pharmacology.

3.2.1. Calcium channel modulation assay.

In vitro calcium channel antagonist and agonist activities were determined using protocols approved by the Health Sciences Animal Welfare Committee at the University of Alberta.

3.2.1.1. Guinea pig ileum longitudinal smooth muscle (GPILSM) calcium channel antagonist assay.

The measurement of CC antagonist receptor activity in smooth muscle requires a stimulus to induce contraction of the muscle that can be either chemical or electrical. Since CC modulators are known to switch pharmacological profile at different voltage,[329] a chemical agonist, such as the muscarinic receptor agonist carbachol (Figure 31) is more appropriate.

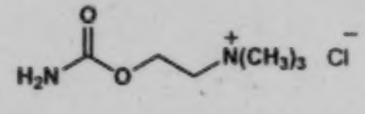


Figure 31: Chemical structure of carbachol chloride.

Smooth muscle CC antagonist activity (IC_{50}) was determined as the molar concentration of the test compound required to produce 50% inhibition of the muscarinic receptor-mediated carbachol (0.167 μM) calcium-dependent contraction (tonic response) of GPILSM. A tissue sample's IC_{50} value was determined graphically, when possible, from the dose-response curve. The average of all samples' IC_{50} values represented the test compound's smooth muscle antagonistic IC_{50} value (± SEM, n = 3).

The simplest model is to suspend the GPILSM in an oxygenated buffer solution in an organ bath, then induce a muscular contraction by adding carbachol to the solution. The tissue contractile force is measured with a force-displacement transducer. After replacing the carbachol containing solution with fresh buffer solution and allowing the tissue to re-equilibrate, the test compound is added and left to perfuse into the tissue. After 10 minutes, carbachol is added to the solution to induce a muscular contraction. The smooth muscle CC antagonist activity is determined as the molar concentration of the test compound required to produce 50% inhibition of the muscarinic receptor-mediated (carbachol, 0.167 μM) calcium-dependent contraction (tonic response) of GPILSM.[22]

A test compound with CC antagonist activity exhibits a weaker carbachol-induced muscular contraction relative to the carbachol-induced contraction in the absence of the test compound.

Calcium channel agonist activity on GPILSM (in the absence of 0.167 μM carbachol) (EC_{50}) was calculated as the micromolar concentration of the test compound required to elicit a 50% increase in the contractile response, relative to the response produced by carbachol (0.167 μM). The average EC_{50} value obtained from three tissue samples provided the test compound's smooth muscle agonist EC_{50} value (± SEM, n = 3).

3.2.1.2. Guinea pig left atrium (GPLA) calcium channel agonist assay.

The key beneficial action of CC agonists is their ability to increase cardiac contractile force without increasing heart rate. The simplest model to measure cardiac CC agonist activity is to suspend a guinea pig atrium in an oxygenated buffer solution, while electrically stimulating the tissue. Because the left atrium is a larger muscle than the right atrium, the right atrium is used to study chronotropic effects, and the left atrium to study inotropic effects. After addition of the test compound in the solution, changes in the cardiac contractile force are observed. The cardiac CC agonist effect is calculated as the percentage increase (positive inotropic effect) in contractile force of isolated guinea pig left atrium (GPLA) relative to its basal contractile force in the absence of the test compound. A compound EC_{50} value was determined graphically, when possible, from the dose-response curve. The average EC_{50} values from three tissue samples was used to calculate the test compound's cardiac positive inotropic EC_{50} value (± SEM, n = 3). Measurements were performed with a force-displacement transducer and recorded on a polygraph machine. The amplitude of each peak determines contractile force, while the number of peaks per time interval determines the heart rate.

All of the 1,4-dihydropyridine calcium channel modulators described in this thesis were racemates.

3.2.2. Nitric oxide release assay.

In vitro nitric oxide release in the presence of either L-cysteine, pig liver esterase, rat serum, or guinea pig serum, determined by quantification of nitrite (NO_2^-) produced by the reaction of nitric oxide with oxygen and water using the Griess reaction (modified procedure described by Nguyen *et al.*),[102] was measured for the test compounds (**67-84**, and **89-94**) and the reference compounds glycerol trinitrate (**38**), O^2-sodium 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (**57**), O^2-sodium 1-[*N*-(2-hydroxyethyl)-*N*-methylamino]diazen-1-ium-1,2-diolate (**113**), O^2-sodium 1-[4-(2-hydroxyethyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (**118**), and O^2-sodium 1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate (**130**), using the following procedure. Phosphate buffer solution (PBS) was prepared by dissolving KH_2PO_4 (50 mM) and $NaHPO_4$ (50 mM) in distilled water. This solution was titrated to physiological pH (7.4) using 0.01 N sodium hydroxide. The buffer containing L-cysteine was prepared in a similar way by dissolving KH_2PO_4 (50 mM), $NaHPO_4$ (50 mM), and L-cysteine (5.0 mM) in distilled water, and then adjusting the pH to 7.4 using a 0.01 N solution of NaOH. Griess reagent was prepared by dissolving sulfanilamide (4.0 g) and *N*-(1-naphthyl)ethylenediamine dihydrochloride (0.2 g) in a mixture of 85% H_3PO_4 (10 mL) and distilled water (approximately 90 mL). An aliquot of 2.4 mL of a 5% dimethyl sulfoxide solution in PBS (10 mL of dimethyl sulfoxide and 190 mL of PBS) was used as blank control solution, which was maintained at 37°C for 1 h with gentle shaking. Griess reagent (0.8 mL) was then added, and the mixture was maintained at 37°C for 30 min with gentle shaking.

A standard nitrite concentration-absorbance plot was prepared by dissolving $NaNO_2$ (0.1 mM) in blank solution, and an aliquot of this solution (2.4 mL) was maintained at 37°C for 1 h with gentle shaking. Griess reagent (0.8 mL) was then added and the mixture was maintained for 30 min at 37 °C with gentle shaking. After incubation, this solution was further diluted by mixing different aliquots with variable volumes of dilution solvent (32 mL of Griess reagent and 96 mL of blank solution). These concentrations of $NaNO_2$ were used to plot a calibration curve from which the nitrite concentration for each test compound was calculated (± SEM, n=3). The percentage nitric oxide released from the test compounds was determined by preparing 0.2 mM solutions in dimethyl sulfoxide. An aliquot (0.12 mL) was diluted with PBS solution (with or without cysteine, 2.28 mL, final concentration 0.1 mM), and then pig liver esterase[240] (suspension in a 3.2 M ammonium sulfate solution, Sigma), rat or guinea pig serum[*] (90 μL), was added. This solution was maintained at 37°C for 1 h with gentle shaking, Griess reagent (0.8 mL) was added, and the mixture was maintained for 30 min at 37 °C with gentle shaking. The ultraviolet absorbance values for the control blank solution, calibration curve, and the test compound absorbance was measured at 540 nm. The absorbance value for each test compound was corrected by subtracting the average blank control absorbance.

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[*] After euthanizing the animals, whole blood was immediately collected from their hearts. The blood was left standing at 5°C for 20 hours so as to permit the serum to separate. The blood was centrifuged at 2000 rpm for 15 minutes and the clear serum was moved into a clean test tube.

The nitrite concentration was determined from the standard nitrite concentration-absorbance curve, which in turn was used to calculate the percentage of nitric oxide released from each test compound.

3.2.3. *In vitro* cyclooxygenase (COX) inhibition assays.

The ability of the test compounds to inhibit ovine COX-1 and COX-2 (IC_{50} value, µM) was determined using an enzyme immuno assay (EIA) kit (catalog no. 560101, Cayman Chemical, Ann Arbor, MI, USA) according to a reported method.[330] Cyclooxygenase catalyzes the first step in the biosynthesis of arachidonic acid (AA) to PGH_2. $PGF_{2\alpha}$, produced from PGH_2 by reduction with stannous chloride, is measured by enzyme immunoassay (ACE competitive EIA). Stock solutions of test compounds were dissolved in a minimum volume of DMSO. Briefly, to a series of supplied reaction buffer solutions (960 µL, 0.1 M Tris-HCl pH 8.0 containing 5 mM EDTA and 2 mM phenol) with either COX-1 or COX-2 (10 µL) enzyme in the presence of heme (10 µL) was added 10 µL of various concentrations of test drug solutions (0.001, 0.01, 0.1, 1, 10, 100 µM in a final volume of 1 mL). These solutions were incubated for a period of 2 min at 37 °C after which 10 µL of AA (100 µM) was added, and the COX reaction was stopped by the addition of 50 µL of 1 M HCl after 2 min. $PGF_{2\alpha}$, was measured by enzyme immunoassay. This assay is based on the competition between PGs and a PG-acetyl cholinesterase conjugate (PG tracer) for a limited amount of PG antiserum. The amount of PG tracer that is able to bind to the PG antiserum is inversely proportional to the concentration of PGs in the wells since the concentration of the PG tracer is held constant while the concentration of PGs varies. This antibody-PG complex binds to a mouse anti-rabbit monoclonal antibody that had been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent, which contains the substrate to acetyl cholinesterase, is added to the well. The product of this enzymatic reaction produces a distinct yellow color that absorbs at 405 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of PG tracer bound to the well, which is inversely proportional to the amount of PGs present in the well during the incubation. Percent inhibition was calculated by comparison of compound treated to various control incubations. The concentration of the test compound causing 50% inhibition (IC_{50}, μM) was calculated from the concentration-inhibition response curve (duplicate determinations).

3.2.4. Antiinflammatory assay.

The test compounds **89-94** and the reference drugs aspirin (**17**), ibuprofen and (**20**) and indomethacin (**22**) were evaluated using the *in vivo* rat carrageenan-induced foot paw edema model reported.[331, 332] *In vivo* antiinflammatory activities were determined after oral administration of aspirin (50, 150 and 250 mg/kg), ibuprofen (50, 150 and 250 mg/kg), indomethacin (3, 10 and 20 mg/kg), or test compounds **89-94** (equivalent doses to those of the parent drugs). All compounds were suspended and administered in 2.0 mL of a 1% methylcellulose solution. Control rats (n = 4) received oral administration of vehicle (2.0 mL of 1.0% methylcellulose solution). Food, but not water, was removed 12 h before administration of test compounds.

3.2.5. Acute ulcerogenesis assay.

The ability to produce gastric damage was evaluated according to a reported procedure.[333] Ulcerogenic activity was evaluated after oral administration of aspirin (250 mg/kg), ibuprofen (250 mg/kg), indomethacin (30 mg/kg) or an equivalent amount of the correspondent test compound (**89-94**). All drugs were suspended and administered orally in 1.7 mL of a 1% methylcellulose solution. Control rats received oral administration of vehicle (1.7 mL of 1.0% methylcellulose solution). Food, but not water, was removed 24 h before administration of test compounds. Six hours after oral administration of the drug, rats were euthanized in a CO_2 chamber and their stomachs were removed, cut out along the greater curvature of the stomach, gently rinsed with water and placed on ice. The number and the length of ulcers were determined using a magnifier lense. The severity of the gastric lesion was measured along its greatest length (1 mm = rating of 1, 1–2 mm = rating of 2, >2 mm = rating according to their length in mm). The average overall length (in mm) of individual ulcers in each tissue was designated as the "ulcer index". Each experimental group consisted of four rats.

3.3. Molecular modeling (docking) studies.

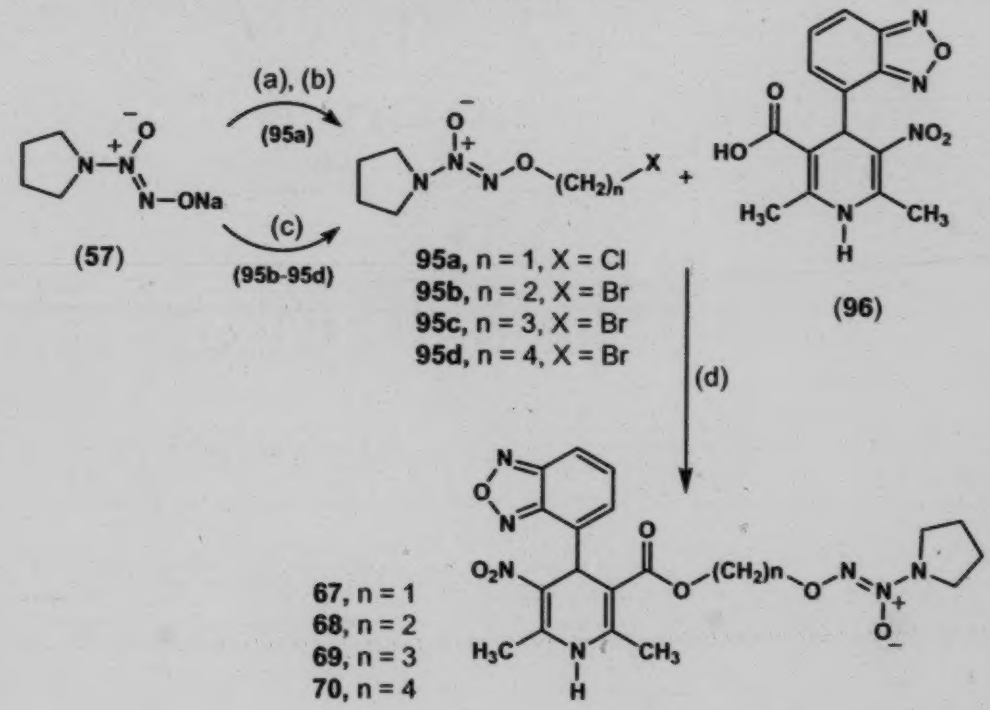
Docking experiments were performed using Insight II software Version 2000.1 (Accelrys Inc.) running on a Silicon Graphics Octane 2 R14000A workstation according to a previously reported method.[330] The coordinates for the X-ray crystal structures of the enzymes COX-1 and COX-2 were obtained from the RCSB Protein Data Bank and hydrogens were added. The ligand molecules were constructed using the Builder module and then energy minimized for 1000 interactions reaching a convergence of 0.01 kcal/mol Å. The docking experiment on COX-2 was carried out by superimposing the energy minimized ligand on SC-558 in the PDB file 1cx2 after which SC-558 was deleted. The resulting ligand enzyme complex was subjected to docking using the Affinity command in the Docking module of Insight II after defining subsets of the enzyme such that residues within 5 Å of the ligand were allowed to relax, while the remainder of the enzyme residues were fixed. The consistent valence force field (CVFF) was employed for all docking purposes. The optimal binding orientation of the ligand-enzyme assembly obtained after docking was further minimized for 1000 interactions using the conjugate gradient method until a convergence of 0.001 kcal/mol Å was reached. The ligand-enzyme assembly was then subjected to a molecular dynamics (MD) simulation using the Discover module Version 2.98 at a constant temperature of 300 K with a 200-step equilibration for over 5000 iterations and a time step of 1 fs using a distance dependent dielectric constant 4*r*.

4.0. Results and discussion.

4.1. Chemistry.

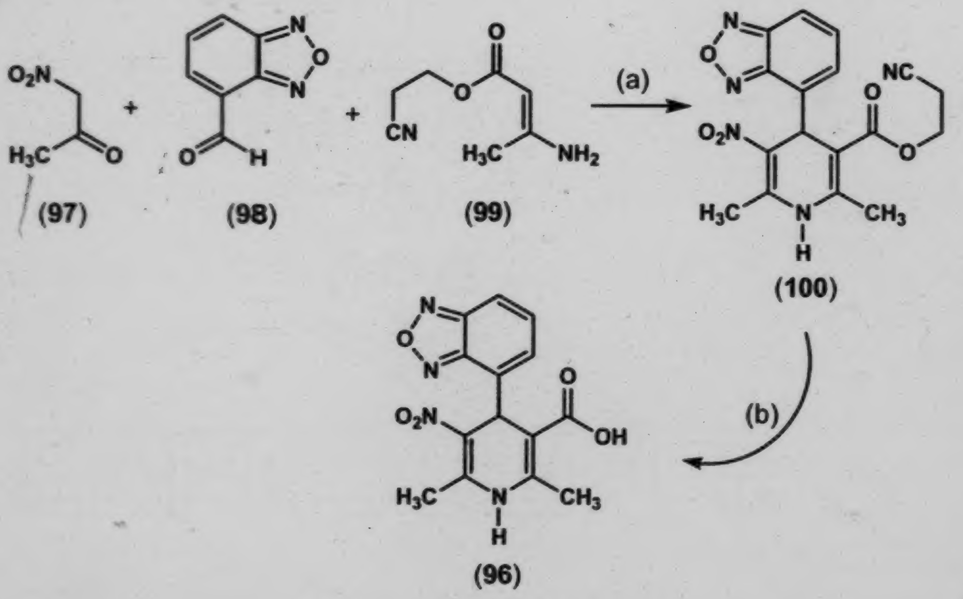
4.1.1. Project 1: synthesis of O^2-alkyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate 1,4-dihydro-2,6-dimethyl-3-nitro-4-(benzofurazan-4-yl)pyridine-5-carboxylates (67-70).

A group of racemic 1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylates possessing a C-4 benzofurazan-4-yl substituent in conjunction with a C-5 O^2-methyl, ethyl, propyl or butyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate ester substituent (**67-70**) were synthesized (Scheme 5).


(57)
(a), (b)
(95a)
(c)
(95b-95d)
95a, n = 1, X = Cl
95b, n = 2, X = Br
95c, n = 3, X = Br
95d, n = 4, X = Br
(96)
(d)
67, n = 1
68, n = 2
69, n = 3
70, n = 4

Scheme 5: Reagents and conditions: (a) $MeSCH_2Cl$, dry DMF/MeCN, 25 °C, 16 h; (b) SO_2Cl_2, dry CH_2Cl_2, 4 °C for 20 min and 25 °C for 1 h; (c) $Br(CH_2)_nBr$, Na_2CO_3, dry DMF/MeCN, 25 °C, 2 h; (d) K_2CO_3, DMF, 25 °C, 48 h.

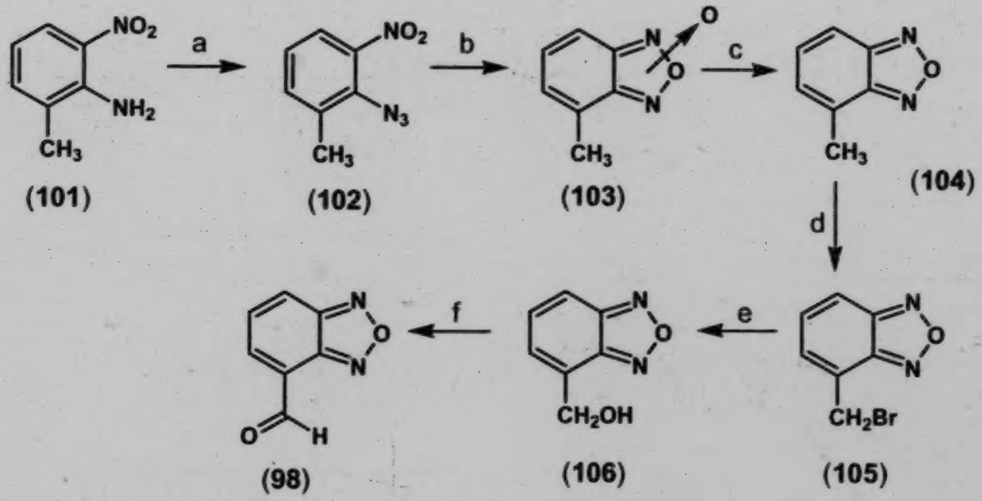
The synthesis of compounds **67-70** started with the preparation of the 1,4-dihydro-2,6-dimethyl-4-(benzofurazan-4-yl)-3-nitropyridine-5-carboxylic acid (**96**), which was obtained by condensation of nitroacetone (**97**), benzofurazan-4-ylcarboxaldehyde (**98**), and 2-cyanoethyl 3-aminocrotonate (**99**), to afford 2-cyanoethyl 1,4-dihydro-2,6-dimethyl-4-(benzofurazan-4-yl)-3-nitro-5-pyridine carboxylate (**100**). The ester **100** was deprotected with DBU (via a base catalyzed β-elimination reaction) at room temperature as shown in Scheme 6.



Scheme 6: reagents and conditions: (a) EtOH, 70 °C, 19 h; (b) DBU, MeOH/H_2O, 25 °C, 19 h.

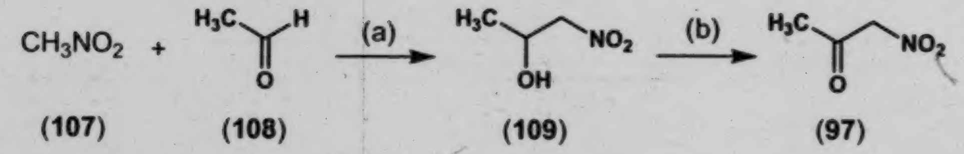
The intermediates **97-99**, which are not commercially available, were prepared as illustrated in Schemes 7-9. Diazotization of 6-methyl-2-nitroaniline (**101**) formed the diazonium salt intermediate which on further reaction with azide anion in a one-pot reaction afforded 2-azido-3-nitrotoluene (**102**). Intramolecular cyclization of **102** in toluene under reflux for 48 h, furnished the 4-methylbenzofuroxan (**103**), which was deoxygenated with sodium azide in ethylene glycol at high temperature (about 140 °C) to yield the benzofurazan derivative (**104**). Bromination of **104** with *N*-bromosuccinimide in carbon tetrachloride, afforded the correspondent 4-(bromomethyl)benzofurazan (**105**), which was reacted under aqueous alkaline conditions to yield 4-(hydroxymethyl)benzofurazan (**106**).

Oxidation of **106** with activated manganese dioxide in chloroform produced the desired benzofurazan-4-yl carboxaldehyde (**98**) in 17% yield overall (Scheme 7).



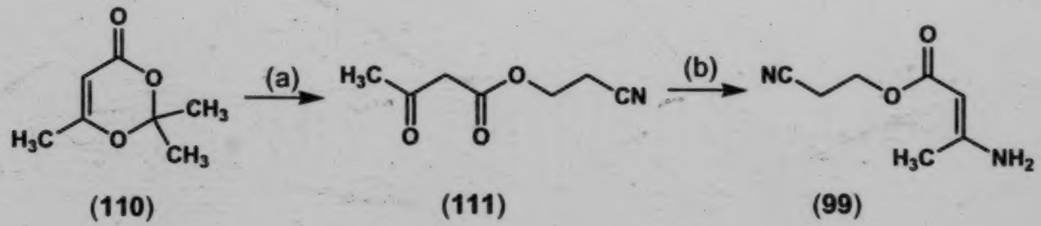
Scheme 7: reagents and conditions: (a) 1. $NaNO_2$, HCl, H_2O, 5 °C, 3h; 2. NaN_3, H_20, 5 °C, 2h; (b) Toluene, reflux, 48h; (c) NaN_3, ethylene glycol, 140 °C, 1h; (d) NBS, CCl_4, reflux, 5h; (e) 1. Na_2CO_3, H_2O/dioxane, reflux, 5h; 2. HCl/H_2O; (f) MnO_2, $CHCl_3$, 25 °C, 24h.

The nucleophilic addition between the conjugated base of nitromethane (**107**) and acetaldehyde (**108**) afforded 1-nitro-2-propanol (**109**), which was oxidized with sodium dichromate at 5 °C for 20 h to obtain nitroacetone (**97**), as illustrated in Scheme 8.



Scheme 8: reagents and conditions: (a) H_2O, Na_2CO_3, 5 °C, 1 h, and 25 °C 19 h; (b) $Na_2Cr_2O_7$, H_2O, H_2SO_4, 5 °C, 20 h.

The last reagent required for the modified Hantzsch reaction, 2-cyanoethyl 3-aminocrotonate (**99**), was synthesized by reaction of 2-cyanoethanol with 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one (**110**) in xylenes, to afford 2-cyanoethyl acetoacetate (**111**), which was converted to 2-cyanoethyl 3-aminocrotonate (**99**) upon reaction with ammonia (gas) in ethyl ether for 19 h at room temperature (Scheme 9).



Scheme 9: reagents and conditions: (a) $NC(CH_2)_2OH$, xylenes, reflux, 72 h; (b) NH_3, ethyl ether, 25 °C, 19 h.

The O^2-(bromoalkyl)-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate intermediates (**95b-95d**) were synthesized in 15 to 17% yield by reaction of O^2-sodium 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (**57**) with 1,2-dibromoethane, 1,3-dibromopropane, or 1,4-dibromobutane, respectively (Scheme 5). Although there are numerous examples in the literature[227] where O^2-sodium *N*-diazeniumdiolates are reported to be versatile nucleophiles that undergo reaction with a variety of electrophilic reagents including alkyl halides, in our hands the sodium diazeniumdiolate (**57**) was a weak nucleophile, since all attempts involving a wide variety of reaction conditions, solvents and reaction times failed to afford chemical yields of products **95b-95d** that were higher than 17%.

The final reaction in the synthesis of the •NO-releasing CC modulators (**67-70**) involved the nucleophilic displacement of the halogen atom (chloride or bromide) present in **95a-95d**, by the potassium carboxylate derived from the acid **96** (Scheme 5). Compounds **67-70** were obtained as yellow powders in moderate yields (20-35 %), after quenching the correspondent reaction upon addition of cold water to the organic solvent.

4.1.1.1. Experimental.

General procedure for the syntheses of O^2-(bromoalkyl)-1-(pyrrolidin-1-yl)diazen-1-ium-1, 2-diolates (95b-95d).

A solution of a dibromoalkane [$Br(CH_2)_nBr$, n = 2, 3 or 4; 41.13 mmol] in MeCN (30 mL) was added to a mixture of O^2-sodium 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (**57**, 5.0 g, 32.65 mmol) and Na_2CO_3 (1.22 g, 11.60 mmol) suspended in DMF (40 mL) and MeCN (20 mL) with stirring at 4 °C under a nitrogen atmosphere. The reaction mixture was allowed to warm to 25 °C, and the reaction was allowed to proceed with stirring for 72 hours, the solvent was partially removed in vacuo, and the mixture was poured onto ice-water (20 mL). Extraction with ether, washing the ether extract with saturated aqueous $NaHSO_3$ solution, drying the ether fraction (Na_2SO_4), and removal of the solvent in vacuo gave a residue that was purified by silica gel column chromatography using EtOAc-hexane (3:7, v/v) as eluent to afford the respective product **95b**, **95c** or **95d**. Some physical and ^1H NMR spectroscopic data for compounds **95b-95d** are listed below. Compounds **95b-95d** were used immediately for the subsequent preparation of compounds **68-70**.

O^2-(2-Bromoethyl)-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (95b).

Compound **95b** was obtained as a yellow oil; 15.5% yield; ^{1}H NMR ($CDCl_3$): δ 1.92-1.95 (m, 4H, pyrrolidinyl H-3, H-4), 3.51-3.5 (m, 6H, 6H, pyrrolidinyl H-2, H-5, BrCH_2), 4.41 (t, J = 6.1 Hz, 2H, CH_2O).

O^2-(3-Bromopropyl)-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (95c).

Compound **95c** was obtained as a yellow oil; 16% yield; ^{1}H NMR ($CDCl_3$): δ 1.93-1.96 (m, 4H, pyrrolidinyl H-3, H-4), 2.27 (quintet, J = 6.1 Hz, 2H, $CH_2CH_2CH_2$); 3.48-3.54 (m, 6H, pyrrolidinyl H-2, H-5, BrCH_2), 4.3 (t, J = 6.1 Hz, 2H, CH_2O).

O^2-(4-Bromobutyl)-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (95d).

Compound **95d** was isolated as a yellow oil; 17.3% yield; ^{1}H NMR ($CDCl_3$): δ 1.86-1.97 (m, 8H, pyrrolidinyl H-3, H-4, $CH_2(CH_2)_2CH_2$), 3.41 (t, J = 6.1 Hz, 2H, BrCH_2), 3.46-3.50 (m, 4H, pyrrolidinyl H-2, H-5), 4.16 (t, J = 6.1 Hz, 2H, CH_2O).

General procedure for the synthesis of *O*^{2}-alkyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate 1,4-dihydro-2,6-dimethyl-3-nitro-4-(benzofurazan-4-yl)pyridine-5-carboxylates (67-70).

A slurry containing the carboxylic acid **96** (0.9 mmol, 1 equiv), the *O*^{2}-haloalkyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate selected (**95a**, **95b**, **95c** or **95d**; 1.08 mmol, 1.2 equiv), and potassium carbonate (1.49 g, 1.08 mmol, 1.2 equiv) in dry DMF (15-25 mL) was stirred at 25 °C for 48 h. The reaction mixture was then poured into ice-water (100 mL), and the mixture was stirred for five minutes. The product was extracted with EtOAc (3 x 50 mL), the EtOAc extract was dried (Na_2SO_4), the solvent was removed in vacuo, and the residue obtained was purified by silica gel column chromatography using EtOAc-hexane (7:3, v/v) as eluent to afford the respective products **67-70**. Physical properties, Infrared (IR), ^{1}H NMR and microanalytical data, for the individual products **67-70** are listed below.

O^2-Methyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate 1,4-dihydro-2,6-dimethyl-3-nitro-4-(benzofurazan-4-yl)pyridine-5-carboxylate (67).

Yellow powder; 35% yield; mp = 177-180 °C; IR (KBr): 3281 (NH), 1716 (CO), 1649 (C=C), 1468 (NO_2), 1213, 756 (N-O); ^{1}H NMR ($CDCl_3$): δ 1.96 (quintet, *J* = 3.9 Hz, 4H, pyrrolidin-1-yl H-3, H-4), 2.30 (s, 3H, C-6 Me), 2.56 (s, 3H, C-2 Me), 3.50 (t, *J* = 3.9 Hz, 4H, pyrrolidyl H-2, H-5), 5.76 (s, 2H, OCH_2O), 5.82 (s, 1H, H-4), 6.90 (s, 1H, N*H*), 7.32 (dd, *J* = 6.3, 8.7 Hz, 1H, benzofurazanyl H-6), 7.44 (d, *J* = 6.3 Hz, benzofurazanyl H-5), 7.65 (d, *J* = 8.7 Hz, 1H, benzofurazanyl H-7). Anal. calcd. for $C_{19}H_{21}N_7O_7$: C, 49.19; H, 4.56; N, 21.13. Found: C, 49.13; H, 4.38; N, 21.01.

O^2-Ethyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate 1,4-dihydro-2,6-dimethyl-3-nitro-4-(benzofurazan-4-yl)pyridine-5-carboxylate (68).

Yellow powder; 20% yield; mp = 163-166 °C; IR (KBr): 3214 (NH), 1703 (CO), 1649 (C=C), 1461 (NO_2), 1213, 756 (N-O); ^{1}H NMR ($CDCl_3$): δ 1.95 (quintet, *J* = 3.9 Hz, 4H, pyrrolidinyl H-3, H-4), 2.36 (s, 3H, C-6 Me), 2.54 (s, 3H, C-2 Me), 3.51 (t, *J* = 3.9 Hz, 4H, pyrrolidinyl H-2, H-5), 4.32-4.36 (m, 4H, OCH_2CH_2O), 5.79 (s, 1H, H-4), 6.33 (s, 1H, N-*H*), 7.38 (dd, *J* = 6.6, 9 Hz, 1H, benzofurazanyl H-6), 7.51 (d, *J* = 6.6 Hz, benzofurazanyl H-5), 7.65 (d, *J* = 9 Hz, 1H, benzofurazanyl H-7). Anal. calcd. for $C_{20}H_{23}N_7O_7$: C, 50.73; H, 4.89; N, 20.70. Found: C, 50.96; H, 4.90; N, 20.64.

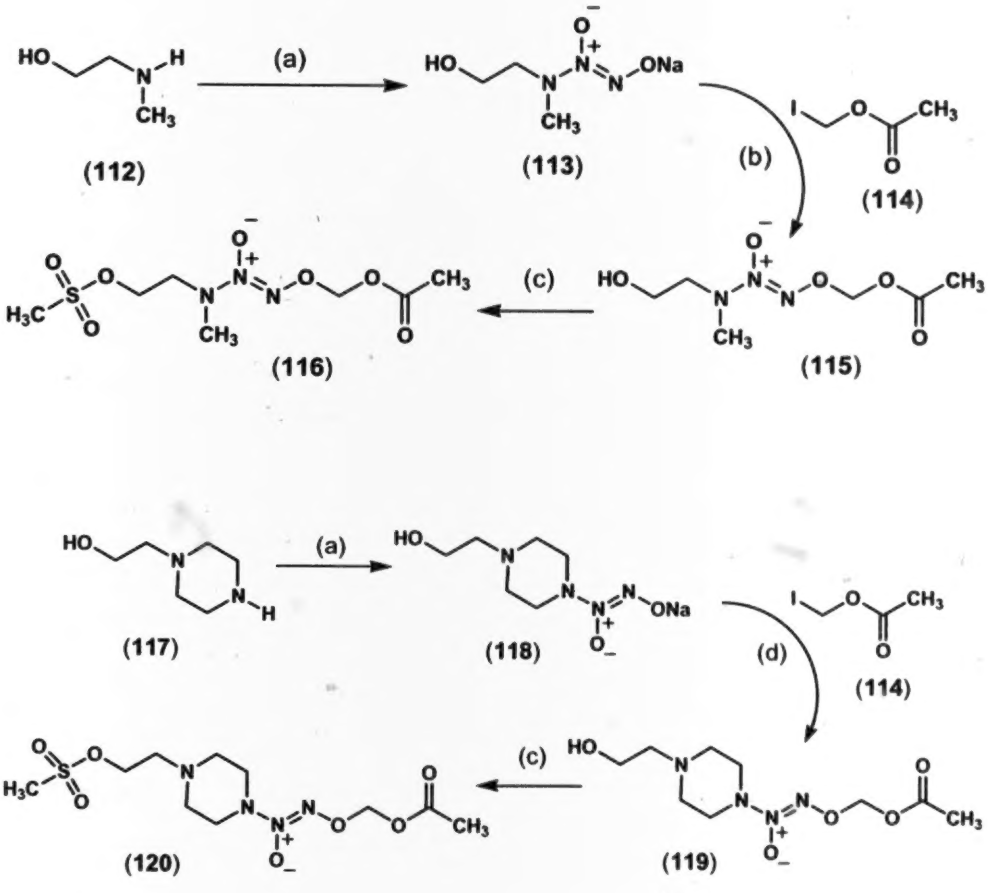
O^2-Propyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate 1,4-dihydro-2,6-dimethyl-3-nitro-4-(benzofurazan-4-yl)pyridine-5-carboxylate (69).

Yellow powder; 20% yield; mp = 157-160 °C; IR (KBr): 3207 (NH), 1710 (CO), 1649 (C=C), 1468 (NO_2), 1219, 756 (N-O); ^{1}H NMR ($CDCl_3$): δ 1.95 (quintet, J = 3.9 Hz, 4H, pyrrolidinyl H-3, H-4), 2.04 (t, J = 6.0 Hz, 2H, $CH_2CH_2CH_2$), 2.38 (s, 3H, C-6 Me), 2.56 (s, 3H, C-2 Me), 3.55 (t, 4H, J = 3.9 Hz, pyrrolidinyl H-2, H-5), 4.13-4.18 (m, 4H, $CH_2CH_2CH_2$), 5.79 (s, 1H, H-4), 6.31 (s, 1H, N*H*), 7.35 (dd, J = 6.3, 8.7 Hz, 1H, benzofurazanyl H-6), 7.45 (d, J = 6.3 Hz, benzofurazanyl H-5), 7.68 (d, J = 8.7 Hz, 1H, benzofurazanyl H-7). Anal. calcd. for $C_{21}H_{25}N_7O_7$: C, 51.26; H, 5.12; N, 19.92. Found: C, 51.38; H, 5.04; N, 19.62.

O^2-Butyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate 1,4-dihydro-2,6-dimethyl-3-nitro-4-(benzofurazan-4-yl)pyridine-5-carboxylate (70).

Yellow powder; 35% yield; mp = 134-136 °C; IR (KBr): 3214 (NH), 1723 (CO), 1649 (C=C), 1481 (NO_2), 1293, 742 (N-O); ^{1}H NMR ($CDCl_3$): δ 1.68-1.73 (m, J = 3.9 Hz, 4H, CH_2CH_2), 1.95 (quintet, J = 3.9 Hz, 4H, pyrrolidinyl H-3, H-4), 2.35 (s, 3H, C-6 Me), 2.54 (s, 3H, C-2 Me), 3.51 (t, J = 3.9 Hz, 4H, pyrrolidinyl H-2, H-5), 4.05 (t, J = 6.1 Hz, 2H, NO-CH_2), 4.15 (t, J = 6.1 Hz 2H, CO_2CH_2), 5.78 (s, 1H, H-4), 6.54 (s, 1H, N*H*), 7.35 (dd, J = 6.6, 9.0 Hz, 1H, benzofurazanyl H-6), 7.43 (d, J = 6.6 Hz, benzofurazanyl H-5), 7.67 (d, J = 9.0 Hz, 1H, benzofurazanyl H-7).

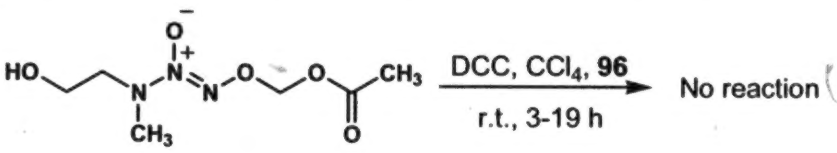
4.1.2. Project 2: synthesis of racemic 4-aryl-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylates possessing an O^2-acetoxymethyl-1-(*N*-ethyl-*N*-methylamino)diazen-1-ium-1,2-diolate (71-75), or O^2-acetoxymethyl-1-(4-ethylpiperazin-1-yl)diazen-1-ium-1,2-diolate (76-80), C-5 ester substituent.



Scheme 10: reagents and conditions: (a) nitric oxide (at 40 psi), CH_3ONa/CH_3OH, ether, 25 °C, 72 h; (b) CH_3CN, 25 °C, overnight; c) CH_3SO_2Cl, DMAP, THF, 25 °C, 15 h; d) THF, -10 °C to 25 °C, 15-18 h.

It was documented that secondary amines react easily with •NO in the presence of sodium methoxide, to form O^2-sodium *N*-diazeniumdiolates. However, there were no reports to our knowledge of aminoalcohols which could be used to attach the •NO-releasing moiety to the 1,4-dihydropyridine CC modulator. In this regard, it was anticipated that the reaction of 2-(*N*-methylamino)ethanol (**112**), or 1-(2-hydroxyethyl)piperazine (**117**), with •NO under the same reaction conditions reported for other secondary amines,[232] would provide the target precursors.

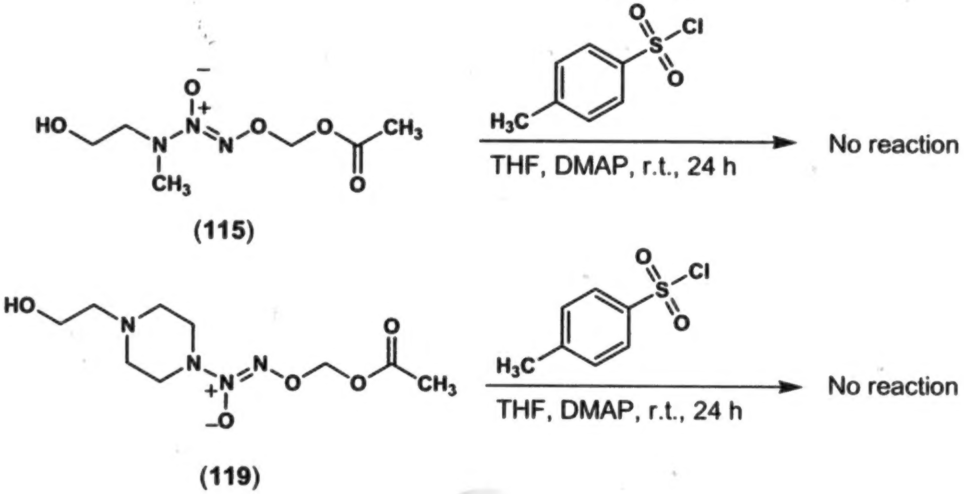
Accordingly, reaction of the amines **112** and **117** with •NO afforded the respective O^2-sodium diazeniumdiolates **113** and **118** (Scheme 10) in 88 and 91 % yield. These O^2-sodium diazeniumdiolates (**113** and **118**) behaved as weak nucleophiles even in the presence of a very good leaving group such as the iodino group of iodomethyl acetate. Thus, reaction of iodomethyl acetate (**114**) with the diazeniumdiolates **113** and **118** afforded the corresponding O^2-acetoxymethyl-protected diazeniumdiolates (**115** and **119**) in 62 and 29 % yield respectively. Chemical intuition suggests the last step for the synthesis of compounds **71-80** should be a simple coupling of the carboxylic acid **96** (shown in Scheme 6, page 71), or **121-124** (shown in Scheme 14, page 82) to the •NO-releasing alcohols **115** and **119**, to form the ester product. It was therefore surprising that the dicyclohexylcarbodiimide (DCC)-catalyzed reaction illustrated in Scheme 11, did not proceed even for a prolonged reaction time of 19 hours.



(115)

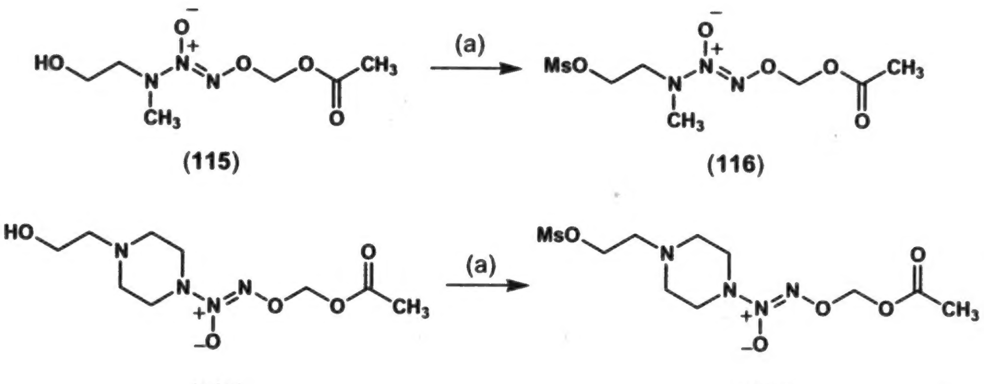
Scheme 11: Attempted coupling reaction between the alcohol **115** and the 1,4-dihydropyridine-3-nitropyridine-5-carboxylic acid (**96**).

This failed attempt to prepare the ester prodrug is attributed to low solubility of compound **115** in carbon tetrachloride. An alternate strategy using a more polar solvent was not pursued since it was decided to prepare the more reactive sulfonate by reaction of the alcohol **115** with toluenesulfonyl chloride as outlined in Scheme 12.



Scheme 12: Preparation of attempted tosylates upon reaction of the alcohols **115** or **119** with *p*-TsCl.

To our surprise, the reaction of compound **115** with tosyl chloride in THF at room temperature, did not show formation of any product on TLC, even when 4-(dimethylamino)pyridine (DMAP) was added to catalyze the reaction. The O^2-acetoxymethylated diazeniumdiolate **119** was also unreactive under similar conditions. These disappointing results prompted the use of the more reactive methanesulfonyl chloride (MsCl). Hence, reaction of compounds **115** and **119** with MsCl in the presence of DMAP in THF afforded quantitative yields of the target mesylates **116** and **120** (Scheme 13) after 19 h of stirring at room temperature.



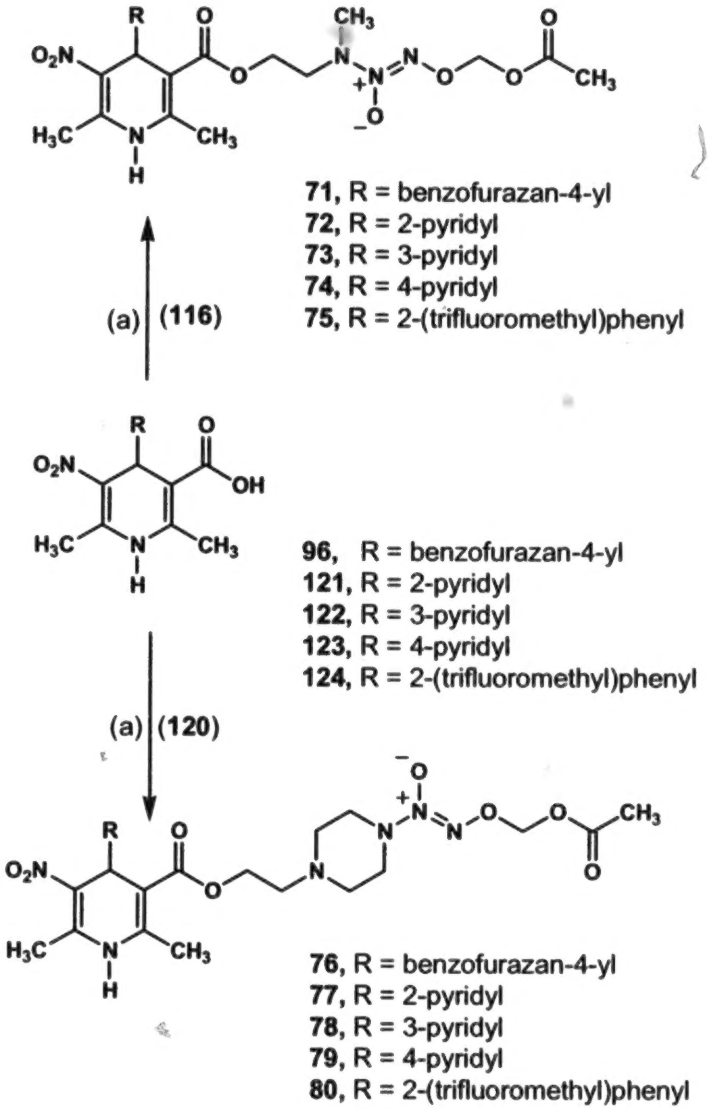
(115) **(116)**

(119) **(120)**

Scheme 13: Reagents and conditions: (a) $ClSO_2CH_3$, DMAP, THF, 25 °C, 19 h.

The final coupling reactions for the synthesis of •NO-releasing CC modulators (**71-80**) proceeded by a nucleophilic displacement (S_N2) of the mesyloxy group present in **116** or **120** by the corresponding sodium 4-aryl-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylate (**96**, **121-124**) in hexamethyl phosphoramide (HMPA) to afford the target products (**71-80**) in moderate yields (19 - 36 %) as illustrated Scheme 14.

Similar reactions using DMF, DMSO, THF, MeCN or different mixtures of these solvents, in place of HMPA, gave lower product yields.



71, R = benzofurazan-4-yl
72, R = 2-pyridyl
73, R = 3-pyridyl
74, R = 4-pyridyl
75, R = 2-(trifluoromethyl)phenyl

96, R = benzofurazan-4-yl
121, R = 2-pyridyl
122, R = 3-pyridyl
123, R = 4-pyridyl
124, R = 2-(trifluoromethyl)phenyl

76, R = benzofurazan-4-yl
77, R = 2-pyridyl
78, R = 3-pyridyl
79, R = 4-pyridyl
80, R = 2-(trifluoromethyl)phenyl

Scheme 14: Reagents and conditions: (a) Na_2CO_3, HMPA, 25 °C, 72 h.

4.1.2.1. Experimental.

Synthesis of iodomethyl acetate (114).

Chloromethyl acetate (108.5 g, 1 mol) was added slowly to a solution of sodium iodide (180 g, 1.2 mol) in dry acetone (600 mL) at 25 °C with stirring. The reaction was allowed to proceed for 3 hours with stirring at 25 °C, the insoluble inorganic salts were removed by filtration, the solvent was evaporated under reduced pressure, the residue was dissolved in dichloromethane (300 mL), and this solution was washed with 2N sodium thiosulfate solution (2 x 100 mL). The organic phase was dried (Na_2SO_4), the solvent was removed *in vacuo* and the residue was purified by fractional distillation (27 °C / 3 mm Hg) to afford iodomethyl acetate (**114**) as a yellow liquid (82.4 g, 41%), which was stored under nitrogen to avoid decomposition prior to use. ^{1}H NMR ($CDCl_3$): δ 5.75 (s, 2H, ICH_2O), 2.11 (s, 3H, CH_3).

O^2-Sodium 1-[*N*-(2-hydroxyethyl)-*N*-methylamino]diazen-1-ium-1,2-diolate (113).

2-(Methylamino)ethanol (**112**, 20 g, 0.26 mol) was added to a solution of sodium methoxide (14.6 g, 0.26 mol, 61 mL of a 25% w/v solution in MeOH) and diethyl ether (300 mL) with stirring at 25 °C. This mixture was flushed with dry nitrogen for five minutes and then the reaction was allowed to proceed under an atmosphere of nitric oxide (40 psi internal pressure) with stirring at 25 °C for 72 h. The product, which precipitated as a fine white powder, was isolated by filtration and then suspended in diethyl ether (100 mL) upon stirring for 15 min. The suspension was filtered, the solid collected was dried at 25 °C under reduced pressure until a constant weight was achieved after about 2 h to afford **113** as a fine white powder (36.1 g, 88 %); mp 150-151 °C; ^{1}H NMR (D_2O) δ 2.86 (t, J = 5.7 Hz, 2H, CH_2CH_2OH), 3.35 (s, 3H, NCH_3), 4.63 (t, J = 5.7

Hz, 2H, CH_2CH_2OH). Product **113** was used immediately after drying without further purification for the synthesis of compound **115**.

O^2-Sodium 1-[4-(2-hydroxyethyl)piperazin-1-yl]-diazen-1-ium-1,2-diolate (118).

Reaction of 1-(2-hydroxyethyl)piperazine (**117**, 20 g, 0.15 mol) under an atmosphere of nitric oxide at 40 psi, using the procedure described for the synthesis of **113** above, afforded **118** (29.5 g, 91%) as a white solid; mp 137-140 °C; ^1H NMR (D_2O) δ 2.61 (t, J = 6.3 Hz, 2H, CH_2CH_2OH), 2.80 (t, J = 4.8 Hz, 4H, piperazin-1-yl H-3, H-5), 3.17 (t, J = 4.8 Hz, 4H, piperazin-1-yl H-2, H-6), 3.73 (t, J = 6.3 Hz, 2H, CH_2CH_2OH). Product **118** was used immediately after drying without further purification for the synthesis of compound **119**.

O^2-Acetoxymethyl-1-[*N*-(2-hydroxyethyl)-*N*-methylamino]diazen-1-ium-1,2-diolate (115).

Freshly distilled iodomethyl acetate (**114**, 27.8 g, 0.14 mol) was added drop wise to a suspension of **113** (20 g, 0.12 mol) in acetonitrile (200 mL, HPLC grade) at 25 °C with stirring. The reaction was allowed to proceed for 19 h at 25 °C with stirring, insoluble inorganic salts were removed by filtration, and the solvent was removed *in vacuo*. DCM was added (150 mL) to the residue, and once again, insoluble inorganic salts were removed by filtration. Removal of the solvent *in vacuo* gave a residue that was purified by silica gel column chromatography using hexane-ethyl acetate, 1:2, v/v) as eluent to afford **115** (15.5 g, 62%) as a pale yellow liquid; ^1H NMR ($CDCl_3$) δ 2.12 (s, 3H, $COCH_3$), 3.10 (s, 3H, NCH_3), 3.50 (t, *J* = 5.1 Hz, 2H, CH_2CH_2OH), 3.78 (t, *J* = 5.1 Hz, 2H, CH_2CH_2OH), 5.78 (s, 2H, OCH_2O). Anal. calcd. for $C_6H_{13}N_3O_5$: C, 34.78; H, 6.32; N, 20.28. Found: C, 34.52; H, 6.68; N, 20.49.

O^2-Acetoxymethyl-1-[4-(2-hydroxyethyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (119).

Freshly distilled iodomethyl acetate (**114**, 20.6 g, 104 mmol) was added dropwise with stirring to a suspension of **118** (20 g, 94 mmol) in dry THF (200 mL) at -50 to -60° C (acetone-dry ice bath). Once the addition was complete, the reaction mixture was allowed to warm to 25 °C, the reaction was allowed to proceed for 19 h, and ethyl acetate (200 mL) was added to quench the reaction. The organic phase was washed with a 0.2 N sodium thiosulphate solution until a pale yellow solution (organic phase) was obtained. The organic phase was dried (Na_2SO_4), the solvent was removed *in vacuo*, and the residue was purified by silica gel column chromatography (ethyl acetate-methanol; 9:1, v/v) to yield **119** (7.34 g, 30%) as a pale yellow liquid; ^{1}H NMR ($CDCl_3$) δ 2.05 (s, 3H, COC*H*$_3$), 2.53 (t, *J* = 5.4 Hz, 2H, C*H*$_2$CH$_2$OH), 2.63 (t, *J* = 5.1 Hz, 4H, piperazin-1-yl *H*-3, *H*-5), 2.82 (s, 1H, O*H*), 3.45 (t, *J* = 5.1 Hz, 4H, piperazin-1-yl *H*-2, *H*-6), 3.58 (t, *J* = 5.4 Hz, 2H, CH$_2$C*H*$_2$OH), 5.71 (s, 2H, OC*H*$_2$O). Anal. calcd. for $C_9H_{18}N_4O_5$: C, 41.22; H, 6.92; N, 21.36. Found: C, 41.02; H, 6.79; N, 21.63.

O^2-Acetoxymethyl-1-[*N*-(2-methylsulfonyloxyethyl)-*N*-methylamino] diazen-1-ium-1,2-diolate (116).

Methanesulfonyl chloride (4.0 g, 35 mmol) in dry THF (50 mL) was added to a solution of 4-dimethylaminopyridine (4.24 g, 35.0 mmol) in dry THF (50 mL) and this mixture was stirred for 10 minutes at 25 °C. A solution of **115** (6.6 g, 32 mmol) in dry THF (90 mL) was added dropwise, and the reaction was allowed to proceed for 15 h at 25 °C with stirring. The solids were filtered off, and the solvent was removed *in vacuo* to give a pale yellow oil which was purified by silica gel column chromatography (hexane-ethyl acetate; 1:2, v/v) to furnish **116** as a colorless oil (3.2 g, 35%); ^{1}H NMR ($CDCl_3$) δ 2.08 (s, 3H, COC*H*$_3$), 3.01 (s, 3H, SO$_2$C*H*$_3$), 3.11 (s, 3H, NC*H*$_3$), 3.69 (t, *J* = 5.1 Hz, 2H, NC*H*$_2$), 4.37 (t, *J* = 5.1

Hz, 2H, CH_2OMs), 5.73 (s, 2H, OCH_2O). Compound **116** was used immediately after purification for the synthesis of **71-75**.

O^2-Acetoxymethyl-1-[4-(2-methylsulfonyloxyethyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (120).

Methanesulfonyl chloride (1.3 g, 11.3 mmol) in dry THF (30 mL) was added to a solution of 4-dimethylaminopyridine (1.37 g, 11.3 mmol) in dry THF (30 mL) and this solution was stirred for 10 minutes at 25 °C. A solution of **119** (2.7 g, 10.3 mmol) in dry THF (30 mL) was added dropwise, the reaction was allowed to proceed for 16 hours at 25 °C with stirring, the solids were filtered off, and the solvent was removed *in vacuo*. The residue obtained was purified by silica gel column chromatography (hexane-ethyl acetate; 1:2, v/v) to afford **120** as a pale yellow oil (2.86 g, 81%); ^{1}H NMR ($CDCl_3$) δ 2.12 (s, 3H, $COCH_3$), 2.72 (t, J = 4.8 Hz, 4H, piperazin-1-yl *H*-3, *H*-5), 2.76 (t, J = 5.1 Hz, 2H, NCH_2), 3.06 (s, 3H, SO_2CH_3), 3.51 (t, J = 4.8 Hz, 4H, piperazin-1-yl *H*-2, *H*-6), 4.33 (t, J = 5.1 Hz, 2H, CH_2OMs), 5.78 (s, 2H, OCH_2O). Compound **120** was used immediately after purification for the synthesis of compounds **76-80**.

General procedure for the synthesis of racemic 4-aryl(heteroaryl)-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylates possessing an O^2-acetoxymethyl-1-(*N*-ethyl-*N*-methylamino)diazen-1-ium-1,2-diolate (71-75), or O^2-acetoxymethyl-1-(4-ethylpiperazin-1-yl)diazen-1-ium-1,2-diolate (76-80), C-5 ester substituent.

A mixture of a 4-aryl-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylic acid (**96**, or **121-124**, 0.47 mmol), sodium carbonate (60 mg, 0.56 mmol) and HMPA (2 mL) was stirred for 4-6 h at 25 °C to form the sodium carboxylate salt. A solution of the mesylate (either **116** or **120,** 0.56 mmol) in HMPA (1 mL) was added, the reaction was allowed to proceed for 72 h at 25 °C with stirring, and then a mixture of ice-water (1:1, v/v; 50 mL) was added to quench the reaction.

Extraction with EtOAc (3 x 30 mL), washing the combined EtOAc extracts with water (5 x 30 mL), drying the EtOAc fraction (Na_2SO_4), and removal of the solvent *in vacuo* gave the respective product (**71-80**) which was purified by silica gel column chromatography (hexane-EtOAc, 1:2, v/v; or EtOAc) and then recrystallization from ether-chloroform. Physical and spectroscopic data for compounds **71-80** are listed below.

O^2-Acetoxymethyl-1-(*N*-methyl-*N*-ethylamino)diazen-1-ium-1,2-diolate 4-(benzofurazan-4-yl)-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylate (71).

Yellow powder; 28% yield; mp 120-122 °C; IR (KBr): 3348 (NH), 1756 (CO), 1649 (C=C), 1474 (NO_2), 1273, 803 (N-O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.05 (s, 3H, $COCH_3$), 2.29 (s, 3H, C-6 CH_3), 2.48 (s, 3H, C-2 CH_3), 2.95 (s, 3H, NCH_3), 3.50-3.55 (m, 2H, CH_2N), 4.12-4.17 (m, 2H, CO_2CH_2), 5.70 (s, 2H, OCH_2O), 5.72 (s, 1H, *H*-4), 6.46 (s, 1H, N*H*), 7.30 (dd, *J* = 8.7, 6.3 Hz, 1H, benzofurazan-4-yl *H*-6), 7.39 (d, *J* = 6.3 Hz, 1H, benzofurazan-4-yl *H*-5), 7.60 (d, *J* = 8.7 Hz, 1H, benzofurazan-4-yl *H*-7). Anal. calcd. for $C_{20}H_{23}N_7O_9$: C, 47.53; H, 4.59; N, 19.40. Found: C, 47.13; H, 4.68; N, 19.05.

O^2-Acetoxymethyl-1-(*N*-methyl-*N*-ethylamino)diazen-1-ium-1,2-diolate 4-(2-pyridyl)-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylate (72).

Yellow powder; 32% yield; mp 133-135 °C; IR (KBr): 3273 (NH), 1776 (CO), 1662 (C=C), 1528 (NO_2), 1212, 829 (N-O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.10 (s, 3H, $COCH_3$), 2.21 (s, 3H, C-6 CH_3), 2.41 (s, 3H, C-2 CH_3), 3.01 (s, 3H, NCH_3), 3.56-3.61 (m, 2H, CH_2CH_2N), 4.14-4.26 (m, 2H, CO_2CH_2), 5.57 (s, 1H, *H*-4), 5.76 (s, 2H, OCH_2O), 7.26 (dd, *J* = 4.5, 6.8 Hz, 1H, pyridyl *H*-5), 7.61 (d, *J* = 7.5 Hz, 1H, pyridyl *H*-3), 7.73 (dd, *J* = 7.5, 6.8 Hz, 1H, pyridyl *H*-4), 8.46 (d, *J* = 4.5Hz, 1H, pyridyl *H*-6), 10.13 (s, 1H, N*H*). Anal. calcd. for $C_{19}H_{24}N_6O_8$: C, 49.14; H, 5.21; N, 18.10. Found: C, 49.14; H, 5.06; N, 17.60.

O^2-Acetoxymethyl-1-(*N*-methyl-*N*-ethylamino)diazen-1-ium-1,2-diolate 4-(3-pyridyl)-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylate (73).

Yellow powder; 21% yield; mp 122-125 °C; IR (KBr): 3288 (NH), 1770 (CO), 1649 (C=C), 1508 (NO_2), 1313, 829 (N-O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.11 (s, 3H, COC*H*$_3$), 2.35 (s, 3H, C-6 C*H*$_3$), 2.51 (s, 3H, C-2 C*H*$_3$), 3.01 (s, 3H, NC*H*$_3$), 3.55-3.63 (m, 2H, C*H*$_2$N), 4.22-4.26 (m, 2H, CO_2C*H*$_2$), 5.35 (s, 1H, *H*-4), 5.76 (s, 2H, OC*H*$_2$O), 7.25 (dd, *J* = 7.8, 4.8 Hz, 1H, pyridyl *H*-5), 7.77 (ddd, *J* = 7.8, 1.8, 1.5 Hz, 1H, pyridyl *H*-4), 8.31 (s, 1H, N*H*), 8.42 (dd, *J* = 4.8, 1.5 Hz, 1H, pyridyl *H*-6), 8.50 (d, *J* = 1.8 Hz, 1H, pyridyl *H*-2). Anal. calcd. for $C_{19}H_{24}N_6O_8$: C, 49.14; H, 5.21; N, 18.10. Found: C, 49.00; H, 5.07; N, 17.87.

O^2-Acetoxymethyl-1-(*N*-methyl-*N*-ethylamino)diazen-1-ium-1,2-diolate 4-(4-pyridyl)-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylate (74).

Yellow powder; 19%; mp 150-152 °C; IR (KBr): 3180 (NH), 1763 (CO), 1649 (C=C), 1494 (NO_2), 1313, 829 (N-O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.11 (s, 3H, COC*H*$_3$), 2.38 (s, 3H, C-6 C*H*$_3$), 2.53 (s, 3H, C-2 C*H*$_3$), 3.00 (s, 3H, NC*H*$_3$), 3.57-3.67 (m, 2H, C*H*$_2$N), 4.23-4.29 (m, 2H, CO_2C*H*$_2$), 5.39 (s, 1H, *H*-4), 5.76 (s, 2H, OC*H*$_2$O), 7.28 (d, *J* = 5.4 Hz, 2H, pyridyl *H*-3, *H*-5), 7.62 (s, 1H, N*H*), 8.48 (d, *J* = 5.4 Hz, 2H, pyridyl *H*-2, *H*-6). Anal. calcd. for $C_{19}H_{24}N_6O_8$: C, 49.14; H, 5.21; N, 18.10. Found: C, 49.05; H, 5.08; N, 17.88.

O^2-Acetoxymethyl-1-(*N*-methyl-*N*-ethylamino)diazen-1-ium-1,2-diolate 4-(2-trifluoromethylphenyl)-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylate (75).

Yellow powder; 36% yield; mp 91-93 °C; IR (KBr): 3328 (NH), 1763 (CO), 1656 (C=C), 1494 (NO_2), 1313, 776 (N-O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.11 (s, 3H, COC*H*$_3$), 2.29 (s, 3H, C-6 C*H*$_3$), 2.46 (s, 3H, C-2 C*H*$_3$), 2.97 (s, 3H, NC*H*$_3$), 3.49-3.57 (m, 1H, CH*H'*N), 3.65-3.75 (m, 1H, C*H*H'N), 4.07-4.15 (m, 1H, CO$_2$CH*H'*), 4.27-4.37 (m, 1H, CO$_2$C*H*H'), 5.75 (s, 2H, OC*H*$_2$O), 5.90 (s, 1H, *H*-4), 7.03 (s, 1H, N*H*), 7.27 (dd, *J* = 6.9, 7.8 Hz, 1H, phenyl *H*-4), 7.40-7.44 (m, 2H, phenyl *H*-5, *H*-6), 7.51 (d, *J* = 7.8 Hz, 1H, phenyl *H*-3). Anal. calcd. for $C_{21}H_{24}F_3N_5O_8$: C, 47.46; H, 4.55; N, 13.18. Found: C, 47.21; H, 4.58; N, 13.06.

***O*^{2}-Acetoxymethyl-1-(4-ethylpiperazin-1-yl)diazen-1-ium-1,2-diolate 4-(benzofurazan-4-yl)-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylate (76).**

Yellow powder; 28% yield; mp 85-88 °C; IR (KBr): 3314 (NH), 1763 (CO), 1649 (C=C), 1501 (NO$_2$), 1266, 803 (N-O) cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 2.13 (s, 3H, COC*H*$_3$), 2.36 (s, 3H, C-6 C*H*$_3$), 2.55 (s, 3H, C-2 C*H*$_3$), 2.53-2.70 (m, 6H, piperazin-1-yl *H*-3, *H*-5, CH$_2$C*H*$_2$N), 3.38-3.45 (m, 4H, piperazin-1-yl *H*-2, *H*-6), 4.16 (t, *J* = 6.3 Hz, 2H, CO$_2$C*H*$_2$), 5.80 (s, 2H, OC*H*$_2$O), 5.81 (s, 1H, *H*-4), 6.66 (s, 1H, N*H*), 7.35 (dd, *J* = 9.0, 6.6 Hz, 1H, benzofurazan-4-yl *H*-6), 7.45 (d, *J* = 6.6 Hz, 1H, benzofurazan-4-yl *H*-5), 7.70 (d, *J* = 9.0 Hz, 1H, benzofurazan-4-yl *H*-7). Anal. calcd. for $C_{23}H_{28}N_8O_9$: C, 49.28; H, 5.04; N, 19.99. Found: C, 49.18; H, 4.99; N, 19.72.

***O*^{2}-Acetoxymethyl-1-(4-ethylpiperazin-1-yl)diazen-1-ium-1,2-diolate 4-(2-pyridyl)-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylate (77).**

Yellow powder; 29% yield; mp 170-171 °C; IR (KBr): 3328 (NH), 1602 (C=C), 1427 (NO$_2$), 1266, 897 (N-O) cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.96 (s, 3H, COC*H*$_3$), 2.19 (s, 3H, C-6 C*H*$_3$), 2.38 (s, 3H, C-2 C*H*$_3$), 2.44 (m, 4H, piperazin-1-yl *H*-3, *H*-5), 2.59 (m, 2H, CH$_2$C*H*$_2$N), 3.18-3.30 (m, 4H, piperazin-1-yl *H*-2, *H*-6), 3.95-4.00 (m, 2H, CO$_2$C*H*$_2$), 5.34 (s, 1H, *H*-4), 5.61 (s, 2H, OC*H*$_2$O), 6.90 (dd, *J* = 6.3, 6.3 Hz, 1H, pyridyl *H*-5), 7.25 (d, *J* = 7.5 Hz, 1H, pyridyl *H*-3), 7.37 (dd, *J* = 7.5, 6.3 Hz, 1H, pyridyl *H*-4), 8.26 (d, *J* = 6.3 Hz, 1H, pyridyl *H*-6), 8.74

(s, 1H, N*H*). Anal. calcd. for $C_{22}H_{29}N_7O_8$: C, 50.86; H, 5.63; N, 18.87. Found: C, 50.46; H, 5.39; N, 18.69.

O^2-Acetoxymethyl-1-(4-ethylpiperazin-1-yl)diazen-1-ium-1,2-diolate 4-(3-pyridyl)-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylate (78).

Yellow powder; 19% yield; mp 75-77 °C; IR (KBr): 3308 (NH), 1763 (CO), 1649 (C=C), 1474 (NO_2), 1273, 742 (N-O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.05 (s, 3H, COC*H*$_3$), 2.38 (s, 3H, C-6 C*H*$_3$), 2.53 (s, 3H, C-2 C*H*$_3$), 2.56-2.64 (m, 6H, piperazin-1-yl *H*-3, *H*-5, CH$_2$C*H*$_2$N), 3.40-3.49 (m, 4H, piperazin-1-yl *H*-2, *H*-6), 4.13-4.19 (m, 2H, CO$_2$C*H*$_2$), 5.39 (s, 1H, *H*-4), 5.79 (s, 2H, OC*H*$_2$O), 7.24 (dd, *J* = 7.5, 4.8 Hz, 1H, pyridyl *H*-5), 7.74 (ddd, *J* = 7.5, 2.1, 1.2 Hz, 1H, pyridyl *H*-4), 8.42 (dd, *J* = 4.8, 1.2 Hz, 1H, pyridyl *H*-6), 8.52 (d, *J* = 2.1 Hz, 1H, pyridyl *H*-2), 8.55 (s, 1H, N*H*). Anal. calcd. for $C_{22}H_{29}N_7O_8$: C, 50.86; H, 5.63; N, 18.87. Found: C, 50.79; H, 5.79; N, 18.65.

O^2-Acetoxymethyl-1-(4-ethylpiperazin-1-yl)diazen-1-ium-1,2-diolate 4-(4-pyridyl)-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylate (79).

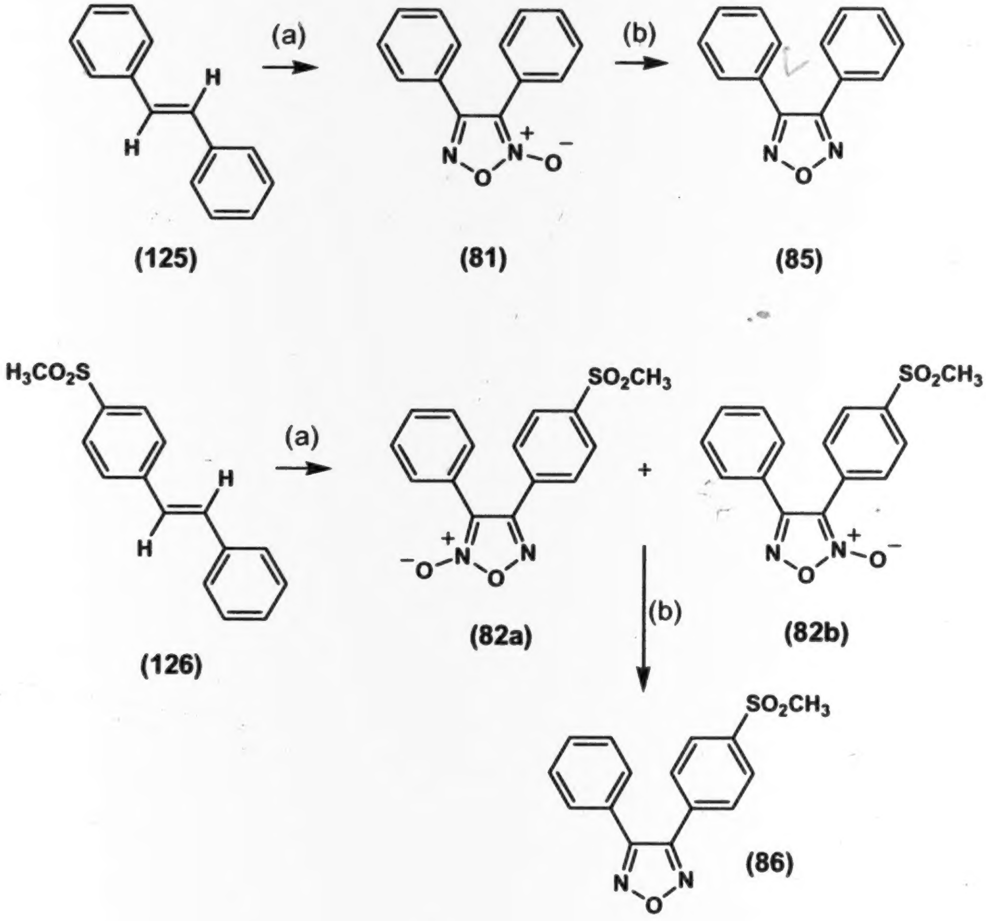
Yellow powder (29%); mp 180-182 °C; IR (KBr): 3053 (NH), 1716 (CO), 1602 (C=C), 1427 (NO_2) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.05 (s, 3H, COC*H*$_3$), 2.36 (s, 3H, C-6 C*H*$_3$), 2.51 (s, 3H, C-2 C*H*$_3$), 2.52-2.61 (m, 6H, piperazin-1-yl *H*-3, *H*-5, CH$_2$C*H*$_2$N), 3.39-3.47 (m, 4H, piperazin-1-yl *H*-2, *H*-6), 4.21-4.27 (m, 2H, CO$_2$C*H*$_2$), 5.36 (s, 1H, *H*-4), 5.79 (s, 2H, OC*H*$_2$O), 7.24 (d, *J* = 5.4 Hz, 2H, pyridyl *H*-3, *H*-5), 7.60 (s, 1H, N*H*), 8.46 (d, *J* = 5.4 Hz, 2H, pyridyl *H*-2, *H*-6). Anal. calcd. for $C_{22}H_{29}N_7O_8$: C, 50.86; H, 5.63; N, 18.87. Found: C, 50.79; H, 5.61; N, 18.63.

O^2-Acetoxymethyl-1-(4-ethylpiperazin-1-yl)diazen-1-ium-1,2-diolate 4-(2-trifluoromethylphenyl)-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylate (80).

Yellow powder; 26% yield; mp 70-72 °C; IR (KBr): 3321 (NH), 1763 (CO), 1649 (C=C), 1501 (NO_2), 1320, 769 (N-O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.12 (s, 3H, COC*H*$_3$), 2.33 (s, 3H, C-6 C*H*$_3$), 2.48 (s, 3H, C-2 C*H*$_3$), 2.54-2.56 (m, 6H, piperazin-1-yl *H*-3, *H*-5, CH_2C*H*$_2$N), 3.37 (m, 4H, piperazin-1-yl *H*-2, *H*-6), 4.06-4.19 (m, 1H, CO_2CH*H'*), 4.21-4.25 (m, 1H, CO_2C*H*H'), 5.78 (s, 2H, OC*H*$_2$O), 5.93 (s, 1H, *H*-4), 6.45 (s, 1H, N*H*), 7.26-7.28 (m, 1H, phenyl *H*-4), 7.40-7.44 (m, 2H, phenyl *H*-3, *H*-5), 7.53 (d, *J* = 7.5 Hz, 1H, phenyl *H*-3). Anal. calcd. for $C_{24}H_{29}F_3N_6O_8$: C, 49.15; H, 4.98; N, 14.33. Found: C, 48.99; H, 4.88; N, 14.13.

4.1.3. Project 3: synthesis of 3,4-diphenyl-1,2,5-oxadiazol-2-oxides (81-84) and 3,4-diphenyl-1,2,5-oxadiazoles (85-88).

Reaction of (*E*)-1,2-diphenylethene (*trans*-stilbene, **125**) with a saturated aqueous solution of sodium nitrite in a mixture of acetic acid and 1,4-dioxane, a modification of a procedure reported by Gasco and co-workers,[334,335] afforded 3,4-diphenyl-1,2,5-oxadiazole-2-oxide (**81**, 26% yield) as illustrated in Scheme 15.



Scheme 15: Reagents and conditions: (a) $NaNO_2$, CH_3CO_2H, 1,4-dioxane, 50-60 °C, 6-17 h; (b) $(EtO)_3P$, reflux, 19-24 h.

A similar reaction using (*E*)-1-[4-(methylsulfonyl)phenyl]-2-phenylethene (**126**) afforded a mixture of the two furoxan regioisomers **82a** and **82b**. This product (**82**) was originally believed to be a single regioisomer since a single spot was observed on micro TLC plates irrespective of the polarity of the development solvent. Subsequent purification by silica gel column chromatography and then recrystallization from hexanes provided a product that showed a single set of resonances in both the ^{1}H NMR and ^{13}C NMR spectra. In order to determine which regioisomer of **82** was isolated, a X-ray crystallographic structure was determined which indicated that this product was a mixture of the two regioisomers 4-[4-(methylsulfonyl)phenyl]-3-phenyl-1,2,5-oxadiazole-2-oxide (**82a**) and 3-[4-(methylsulfonyl)phenyl]-4-phenyl-1,2,5-oxadiazole-2-oxide (**82b**) that were present in a 3:1 ratio (Figure 32). Reaction of furoxan **81**, or the regioisomers **82a-82b**, with triethylphosphite at reflux for 19-24 hours afforded the respective deoxygenated product 3,4-diphenyl-1,2,5-oxadiazole (**85**, 70%), or 3-[4-(methylsulfonyl)phenyl]-4-phenyl-1,2,5-oxadiazole (**86**, 84%).

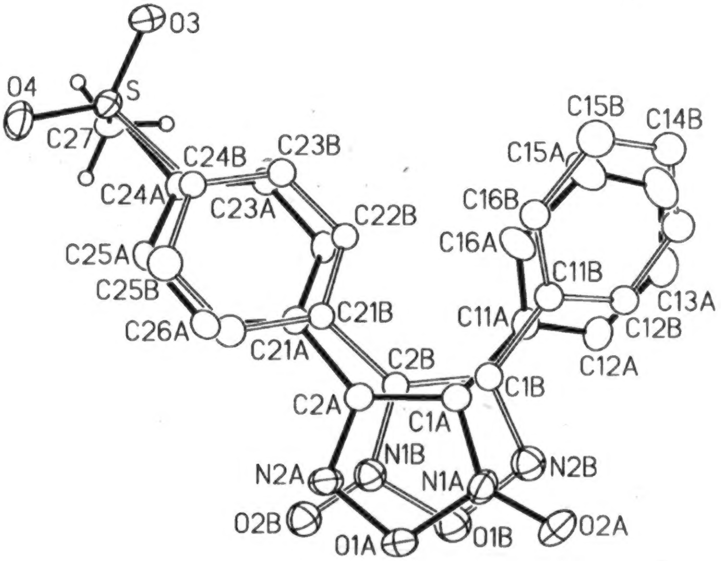
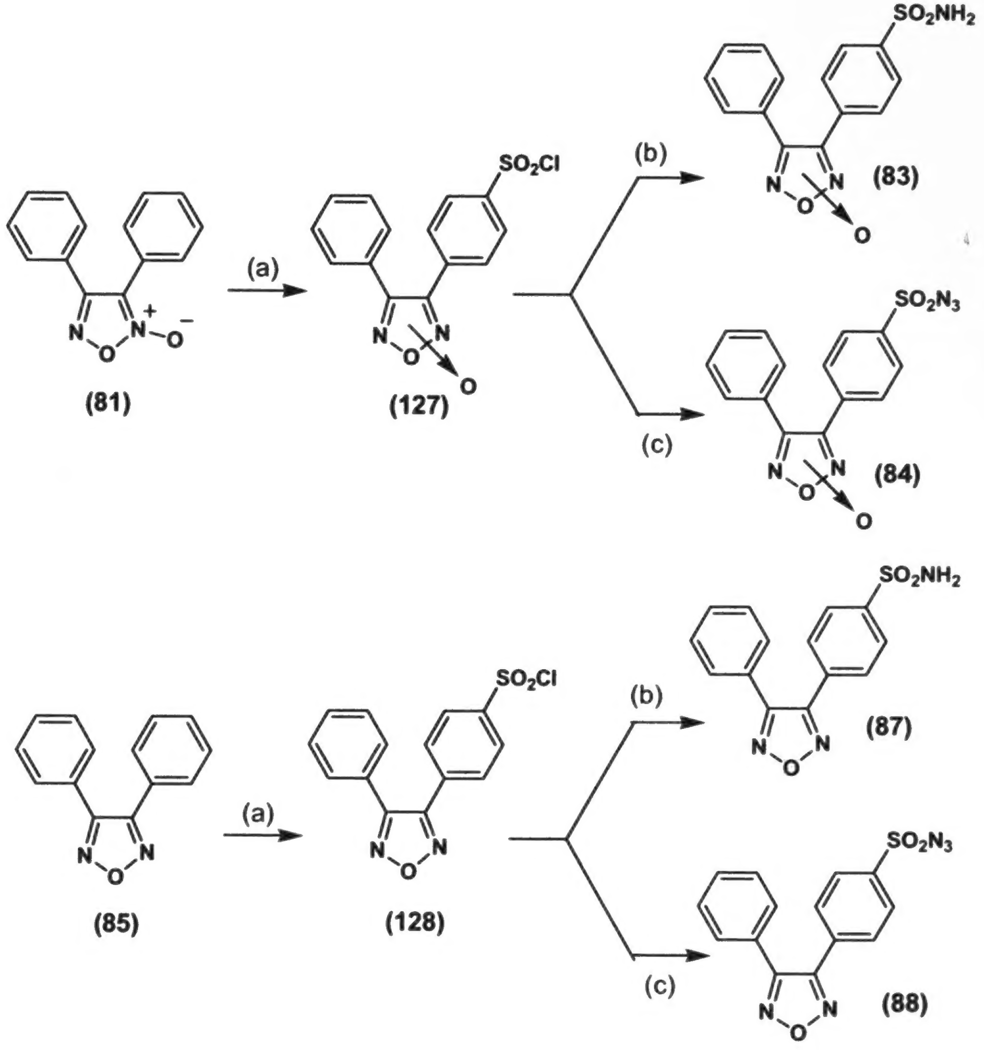

O3
O4
S
C27
C24B
C23B
C24A
C23A
C22B
C25A
C25B
C26A
C21B
C21A
C2B
C2A
C1A
N1B
N2A
N1A
O2B
O1A
O1B
O2A
N2B
C1B
C15B
C14B
C15A
C16B
C16A
C11B
C11A
C13A
C12B
C12A

Figure 32: X-ray crystal structure of a mixture (3:1 ratio) of the 4-[4-(methylsulfonyl)phenyl]-3-phenyl-1,2,5-oxadiazole-2-oxide (**82a**), and 3-[4-(methylsulfonyl)phenyl]-3-phenyl-1,2,5-oxadiazole-2-oxide (**82b**), regioisomers. Atom labels ending in "A" (e.g. O1A, N1A, C1A) belong to **82a**, while those ending in "B" (O1B, N1B, C1B) belong to **82b** (the $MeSO_2$ group is common to both).

Chlorosulfonation of the unsymmetrical furoxan **81** with chlorosulfonic acid at 25 °C for 17 hours afforded the sulfonyl chloride **127** that was purified by silica gel column chromatography to provide a 30% isolated yield. Although the ^1H NMR spectrum for the sulfonyl chloride product **127** showed a single set of resonances similar to **82a-82b**, it may also exist as a mixture of the regioisomers 4-[4-(chlorosulfonyl)phenyl]-3-phenyl-1,2,5-oxadiazole-2-oxide and 3-[4-(chlorosulfonyl)phenyl]-4-phenyl-1,2,5-oxadiazole-2-oxide) as shown in Scheme 16.

A similar chlorosulfonation of the symmetrical furazan **85** furnished 3-[4-(chlorosulfonyl)phenyl]-4-phenyl-1,2,5-oxadiazole (**128**), which unlike **127** can not exist as a mixture of two regioisomers, in 16% isolated yield after purification by silica gel column chromatography. The subsequent reaction of the sulfonyl chloride **127** with NH_4OH afforded the sulphonamide product (**83**, presumed to be a mixture of two regioisomers, 69%). Alternatively, reaction of **127** with the nucleophile NaN_3 yielded the respective sulfonylazide product (**84**, 76%) that is assumed to exist as a mixture of two regioisomers. Similar reactions of the sulfonyl chloride **128** with NH_4OH furnished 3-[4-(aminosulfonyl)phenyl]-4-phenyl-1,2,5-oxadiazole (**87**, 82%), or with NaN_3 yielded 3-[4-(azidosulfonyl)phenyl]-4-phenyl-1,2,5-oxadiazole (**88**, 86%).


SO₂NH₂
(a)
(b)
(c)
(81)
(127)
SO₂Cl
(83)
SO₂N₃
(84)
SO₂NH₂
SO₂Cl
(85)
(128)
(87)
SO₂N₃
(88)
N
O

Scheme 16: Reagents and conditions: (a) $ClSO_3H$, 25 °C, 17 h; (b) NH_4OH, THF, 25 °C, 1.5 h; (c) NaN_3, H_2O, THF, 25 °C, 20 h.

4.1.3.1. Experimental.

3,4-Diphenyl-1,2,5-oxadiazole-2-oxide (81).

trans-Stilbene (**125**, 2.0 g, 11 mmol) was dissolved in a warm mixture (50-55 °C) of glacial acetic acid (6 mL) and 1,4-dioxane (10 mL). Aqueous sodium nitrite (5.34 g, 77.6 mmol in 10 mL of water) was added dropwise during a period of 1 h, the reaction was allowed to proceed with stirring at 50-60 °C for 6.5 h, and the reaction was quenched by addition of ice-water (100 mL of a 50:50 v/v mixture) at 25 °C. This mixture was extracted with EtOAc (3 x 30 mL), the organic phase was dried (Na_2SO_4), and the solvent was removed *in vacuo*. The residue obtained was purified by silica gel column chromatography (hexanes-ether; 3:1 v/v) prior to recrystallization from hexanes to yield **81** (0.63 g, 26%) as pale yellow crystals; mp 104-105 °C; IR (KBr): 3059 (CH), 1588 (NO), 1420 (NO) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.43-7.55 (m, 10H, phenyl hydrogens). Anal. calcd. for $C_{14}H_{10}N_2O_2$: C, 70.58; H, 4.23; N, 11.76. Found: C, 70.33; H, 4.19; N, 11.61.

4-[4-(Methylsulfonyl)phenyl]-3-phenyl-1,2,5-oxadiazole-2-oxide (82a) and 3-[4-(methylsulfonyl)phenyl]-4-phenyl-1,2,5-oxadiazole-2-oxide (82b) regioisomers (ratio 3:1).

(*E*)-1-[4-(Methylsulfonyl)phenyl]-2-phenylethene (**126**, 1.12 g, 4.3 mmol) was dissolved in a warm mixture (55-60 °C) of glacial acetic acid (5 mL) and 1,4-dioxane (50 mL). Aqueous sodium nitrite (2.2 g, 32 mmol in 4 mL of water) was added dropwise during a period of 1 h, the reaction was allowed to proceed with stirring at 60 °C for 17 h, and the reaction was quenched by addition of ice-water (100 mL of a 50:50 v/v mixture) at 25 °C. This mixture was extracted with DCM (3 x 40 mL), the organic extract was dried (Na_2SO_4), and the solvent was removed *in vacuo*. The residue obtained was purified by silica gel column chromatography (hexanes:$CHCl_3$:EtOAc; 50:35:15 v/v/v) to yield a mixture of the two regioisomers **82a** and **82b** that were subsequently shown by X-ray crystallography to be present in a ratio of 3:1 (0.65 g, 47%) as pale yellow crystals; mp 121-123 °C; IR (KBr): 3059 (CH_{arom}), 2918 (CH_{aliph}), 1595 (NO), 1313 (SO) cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.12 (s, 3H, CH_3), 7.43-7.40 (m, 5H, phenyl hydrogens), 7.68 (d, J = 8.4 Hz, 2H, 4-methylsulfonylphenyl *H*-2, *H*-6), 7.96 (d, J = 8.4 Hz, 2H, 4-methylsulfonylphenyl *H*-3, *H*-5). Anal. calcd. for $C_{15}H_{12}N_2O_4S$: C, 56.95; H, 3.82; N, 8.86. Found: C, 56.96; H, 3.76; N, 8.89.

Crystal structure data for the 4-[4-(methylsulfonyl)phenyl]-3-phenyl-1,2,5-oxadiazole-2-oxide (82a) and 3-[4-(methylsulfonyl)phenyl]-4-phenyl-1,2,5-oxadiazole-2-oxide (82b) mixture or regioisomers (3:1 ratio).

Molecular formula: $C_{15}H_{12}N_2O_4S$, formula weight: 316.33, crystal system: monoclinic, space group: $P2_1/c$ (No. 14) with unit cell dimensions a = 18.1969 (18) Å, b = 7.1642 (7) Å, c = 11.6272 (11) Å, β = 106.4720 (17)°, V = 1453.6 (2) $Å^3$, Z = 4, ρ_{calcd} = 1.445 g cm^{-3}, μ = 0.242 mm^{-1}. A crystal fragment of approximate dimensions (mm^3) 0.49 x 0.25 x 0.04 was mounted in a non-specific orientation on a Bruker PLATFORM/SMART 1000 CCD diffractometer. All intensity measurements were performed using Mo Kα radiation (λ = 0.71073 Å) with a graphite crystal incident beam monochromator. The intensity data were collected at -80° using ω scans (0.2° scans, 45 s exposures). A total of 2970 independent reflections were collected to a maximum 2θ limit at 52.86°. The structure was solved by direct methods (*SHELXS-86*). Refinement of atomic parameters was carried out by using full-matrix least-squares on F^2 (*SHELXL-93*). Although the initial model refined was for **82a**, elongation of the thermal parameters for the phenyl and oxadiazole rings, plus the occurrence of a large residual electron-density peak near the 5-nitrogen of the 1,2,5-oxadiazole ring, suggested that the isomer **82b** was also present. The two isomers share the $MeSO_2$ group, but separate sets of positions for the phenyl and 1,2,5-oxadiazole 2-oxide groups were refined for both **82a** and **82b**, in 75:25 ratio. The final model gave agreement factors (R indices) of $R_1(F)$ =

0.0685 (for 2432 data with $I \geq 2\sigma(I)$) and $wR_2(F^2) = 0.1982$ (for all 2970 unique data). Crystallographic data (excluding structure factors) have been deposited at the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 252139. Copies of the data can be obtained free of charge by application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (Fax: 44-0-1223-336033 or, e-mail: deposit@ccdc.cam.ac.uk, or http://www.ccdc.cam.uk).

3,4-Diphenyl-1,2,5-oxadiazole (85).

A solution of **81** (0.3 g, 1.2 mmol) in triethylphosphite (10 mL) was heated at reflux for 24 h with stirring, the solution was cooled to 25 °C, and the reaction mixture was stirred for 25 °C for 2 h. The reaction was quenched by addition of H_2SO_4 (100 mL of 2 N), the mixture was stirred for 20 minutes, and the product was extracted with DCM (2 x 50 mL). The combined DCM extracts were washed with water (6 x 50 mL), the organic phase was dried (Na_2SO_4), and the solvent was removed *in vacuo*. The residue obtained was purified by silica gel column chromatography (hexanes-ether, 4:1 v/v) to yield **85** (0.19 g, 70 %) as a white powder; mp 67-70 °C; ^{1}H NMR ($CDCl_3$) δ 7.41-7.56 (m, 10H, phenyl hydrogens). Anal. calcd. for $C_{14}H_{10}N_2O$: C, 75.66; H, 4.54; N, 12.60. Found: C, 75.39; H, 4.80; N, 12.78.

3-[4-(Methylsulfonyl)phenyl]-4-phenyl-1,2,5-oxadiazole (86).

A mixture (ratio of 3:1) of the two regioisomers **82a** and **82b** (0.65 g, 2.05 mmol) dissolved in triethylphosphite (10 mL) was heated at reflux for 24 h with stirring. The reaction mixture was cooled to 25 °C, the reaction was allowed to proceed at 25 °C for an additional 1 h with stirring, and the reaction was quenched by addition of H_2SO_4 (10 mL of 2 N) with subsequent stirring for 20 minutes. This mixture was extracted with DCM (3 x 30 mL), the combined DCM extracts were washed with water (6 x 30 mL), the organic fraction was dried (Na_2SO_4), and the solvent was removed *in vacuo*. The residue obtained was purified by silica gel column chromatography (hexanes-ether; 3:1 v/v) to yield **86** (0.51 g, 84%) as a white powder; mp 135-137 °C; IR (KBr): 3012 (CH_{arom}), 2925 (CH_{aliph}), 1595 (NO), 1313 (SO) cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.12 (s, 3H, CH_3), 7.56-7.48 (m, 5H, phenyl hydrogens), 7.78 (d, J = 8.4 Hz, 2H, 4-methylsulfonylphenyl *H*-2, *H*-6), 8.03 (d, J = 8.4 Hz, 2H, 4-methylsulfonylphenyl *H*-3, *H*-5). Anal. calcd. for $C_{15}H_{12}N_2O_3S$: C, 59.99; H, 4.03; N, 9.33. Found: C, 59.71; H, 4.01; N, 9.09.

4-[4-(Chlorosulfonyl)phenyl]-3-phenyl-1,2,5-oxadiazole-2-oxide and 3-[4-(chlorosulfonyl)phenyl]-4-phenyl-1,2,5-oxadiazole-2-oxide (127) regioisomers.

3,4-Diphenyl-1,2,5-oxadiazole-2-oxide (**81**, 0.5 g, 2 mmol) was added slowly to a solution of chlorosulfonic acid (5 mL) at ice-bath temperature, the reaction mixture was stirred at this temperature for 10 minutes, the ice bath was removed, and the reaction was allowed to proceed at 25 °C for 17 h. The brown reaction mixture was poured dropwise with caution onto crushed ice (100 g). Extraction with EtOAc (3 x 30 mL), repeated washing of the EtOAc extract with water until the water wash achieved a neutral pH of 7, drying the EtOAc fraction (Na_2SO_4), and the solvent was removed *in vacuo*. The residue obtained was purified by silica gel column chromatography (hexanes-ether, 1:1 v/v) to afford **127** (0.21 g, 30%) as a pale brown liquid; ^1H NMR ($CDCl_3$) δ 7.50-7.53 (m, 5H, phenyl hydrogens), 7.83 (d, J = 8.4 Hz, 2H, 4-chlorosulfonylphenyl *H*-2, *H*-6), 8.10 (d, J = 8.4 Hz, 2H, 4-chlorosulfonylphenyl *H*-3, *H*-5). The sulfonyl chloride product **127** was used immediately after purification for the synthesis of compounds **83** and **84**.

3-[4-(Chlorosulfonyl)phenyl]-4-phenyl-1,2,5-oxadiazole (128).

3,4-Diphenyl-1,2,5-oxadiazole (**85**, 0.86 g, 3.87 mmol) was added to chlorosulfonic acid (5 mL) at ice-bath temperature with stirring, after 10 minutes the ice bath was removed, the reaction mixture was allowed to warm to 25 °C, and the reaction was allowed to proceed at 25 °C for 17 h. The brown reaction mixture was poured dropwise with caution onto crushed ice (100 g). The product was extracted with EtOAc (3 x 30 mL), and the organic phase was washed repeatedly with water until the pH was neutral. The EtOAc fraction was dried (Na_2SO_4), the solvent was removed *in vacuo*, and the residue obtained was purified by silica gel column chromatography (hexanes-EtOAc, 96:4 v/v) to furnish the sulfonyl chloride product **128** (0.20 g, 16%) as a pale brown liquid; ^{1}H NMR ($CDCl_3$) δ 7.50-7.52 (m, 5H, phenyl hydrogens), 7.84 (d, *J* = 8.1 Hz, 2H, 4-chlorosulfonylphenyl *H*-2, *H*-6), 8.11 (d, *J* = 8.1 Hz, 2H, 4-chlorosulfonylphenyl *H*-3, *H*-5). The sulfonyl chloride **128** was used immediately after purification for the synthesis of compounds **87** and **88**.

4-[4-(Aminosulfonyl)phenyl]-3-phenyl-1,2,5-oxadiazole-2-oxide and 3-[4-(aminosulfonyl)phenyl]-4-phenyl-1,2,5-oxadiazole-2-oxide regioisomers (83).

An excess of ammonium hydroxide (2 mL of 30% w/v) was added dropwise to a mixture of the sulfonyl chloride regioisomers **127** (0.24 g, 0.71 mmol) in THF (10 mL) at 25 °C with stirring. The reaction was allowed to proceed for 1.5 h at 25 °C, water (100 mL) was added to quench the reaction, and this mixture was extracted with EtOAc (3 x 20 mL). The combined EtOAc extracts were washed with water (3 x 25 mL) and the EtOAc fraction was dried (Na_2SO_4). Removal of the solvent *in vacuo* afforded **83** (0.15 g, 69%) as a white powder; mp 245-247 °C; IR (KBr): 3321 (NH), 3261 (NH), 1595 (NO), 1326 (SO) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 6.70 (s, 2H, N*H*$_2$), 7.31-7.37 (m, 5H, phenyl hydrogens), 7.47 (d, *J* = 8.4 Hz, 2H, 4-aminosulfonylphenyl *H*-2, *H*-6), 7.82 (d, *J* = 8.4 Hz, 2H, 4-aminosulfonylphenyl *H*-3, *H*-5). Anal. calcd. for $C_{14}H_{11}N_3O_4S$: C, 52.99; H, 3.49; N, 13.24. Found: C, 53.31; H, 3.34; N, 13.15.

4-[4-(Azidosulfonyl)phenyl]-3-phenyl-1,2,5-oxadiazole-2-oxide and 3-[4-(azidosulfonyl)phenyl]-4-phenyl-1,2,5-oxadiazole-2-oxide regioisomers (84).

Sodium azide (87 mg, 1.26 mmol) was added to a mixture of regioisomers **127** (0.21 g, 0.63 mmol) in THF (10 mL) and water (2 mL) at 25 °C. The reaction was allowed to proceed for 12 h with stirring at 25 °C, EtOAc (40 mL) was added to quench the reaction, the insoluble solid was removed by filtration and discarded, the organic phase was washed with water (2 x 15 mL), dried (Na_2SO_4), and the solvent was removed *in vacuo*. The residue obtained was purified by silica gel column chromatography (hexanes-ether, 3:1 v/v) to furnish **84** (0.16 g, 76%) as white crystals; mp 85-90 °C; IR (KBr): 3059 (CH), 2146 (N_3), 1595 (NO), 1380 (SO) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.56-7.59 (m, 5H, phenyl hydrogens), 7.82 (d, *J* = 8.4 Hz, 2H, 4-azidosulfonylphenyl *H*-2, *H*-6), 8.04 (d, *J* = 8.4 Hz, 2H, 4-azidosulfonylphenyl *H*-3, *H*-5). Anal. calcd. for $C_{14}H_9N_5O_4S$: C, 48.98; H, 2.64; N, 20.40. Found: C, 49.16; H, 2.40; N, 20.43.

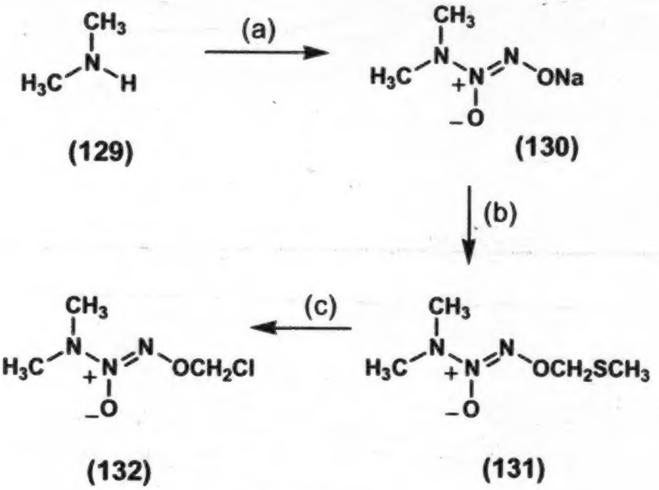
3-[4-(Aminosulfonyl)phenyl]-4-phenyl-1,2,5-oxadiazole (87).

Excess ammonium hydroxide (1 mL of 30% w/v) was added dropwise to a solution of the sulfonyl chloride **128** (81 mg, 0.25 mmol) in THF (10 mL) at 25 °C. The reaction was allowed to proceed for 1.5 h at 25 °C with stirring and water (100 mL) was added to quench the reaction. Extraction with EtOAc (3 x 20 mL), washing the combined EtOAc extracts with water (3 x 25 mL), drying the EtOAc fraction (Na_2SO_4), and removal of the solvent *in vacuo* yielded **87** (62 mg, 82%) as a white powder; mp 227-229 °C; IR (KBr): 3314 (NH), 3224 (NH), 1333 (SO) cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.76 (s, 2H, NH_2), 7.23-7.27 (m, 5H, phenyl hydrogens), 7.39 (d, *J* = 8.4 Hz, 2H, 4-aminosulfonylphenyl *H*-2, *H*-6), 7.74 (d, *J* = 8.4 Hz, 2H, 4-aminosulfonylphenyl *H*-3, *H*-5). Anal. calcd. for $C_{14}H_{11}N_3O_3S$: C, 55.80; H, 3.68; N, 13.95. Found: C, 55.99; H, 3.59; N, 13.75.

3-[4-(Azidosulfonyl)phenyl]-4-phenyl-1,2,5-oxadiazole (88).

Sodium azide (72 mg, 1.11 mmol) was added to the sulfonyl chloride **128** (0.11 g, 0.37 mmol) in THF (10 mL) and water (2 mL) at 25 °C with stirring. The reaction was allowed to proceed for 12 h with stirring at 25 °C, EtOAc (40 mL) was added to quench the reaction, the solids removed by filtration were discarded, the organic phase was washed with water (2 x 15 mL), the organic phase was dried (Na_2SO_4), and the solvent was removed *in vacuo*. The residue obtained was purified by silica gel column chromatography (hexanes-EtOAc, 90:10 v/v) to afford **88** (0.10 g, 86%) as a white semi-solid; IR (KBr): 3073 (CH), 2139 (N_3), 1595 (NO), 1434 (SO); ^{1}H NMR ($CDCl_3$) δ 7.46-7.51 (m, 5H, phenyl hydrogens), 7.71 (d, *J* = 8.4 Hz, 2H, 4-azidosulfonylphenyl *H*-2, *H*-6), 8.07 (d, *J* = 8.4 Hz, 2H, 4-azidosulfonylphenyl *H*-3, *H*-5). Anal. calcd. for $C_{14}H_9N_5O_3S$: C, 51.37; H, 2.77; N, 21.40. Found: C, 51.53; H, 2.45; N, 21.27.

4.1.4. Project 4: synthesis of •NO-NSAIDs (**89-94**).

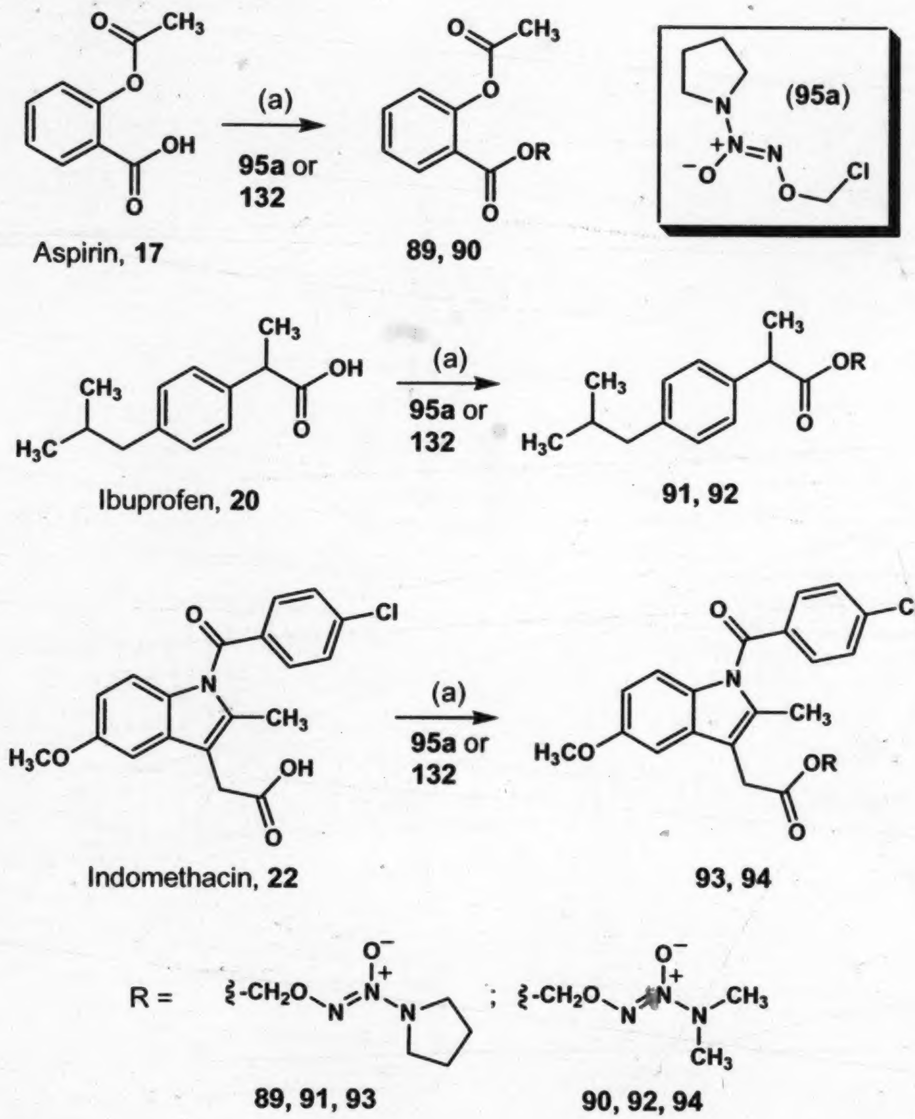


Scheme 17: Reagents and conditions: (a) nitric oxide (40 psi), $NaOCH_3$, CH_3OH, ether, 25 °C, 19 h; (b) $ClCH_2SCH_3$, HMPA, 25 °C, 48 h; (c) SO_2Cl_2, CH_2Cl_2, 25 °C, 3 h.

O^2-Chloromethyl-1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate (**132**) was prepared according to a modified procedure reported by Tang *et. al*,[241] as illustrated in Scheme 17. Thus, reaction of dimethylamine (**129**) with •NO gas (40 psi) at room temperature in the presence of sodium methoxide, afforded O^2-sodium 1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate (**130**) in 90% yield. The sodium salt was alkylated with chloromethyl methyl sulfide to afford O^2-(methylthiomethyl)-1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate (**131**), which was subsequently reacted with sulfuryl chloride in DCM for 4 h to afford the O^2-chloromethyl-protected diazeniumdiolate **132** in quantitative yield.

The target •NO-NSAID ester prodrugs **89-94** were synthesized in moderate-to-good yields (40-81%) by condensation of the sodium salt of acetylsalicylic acid (**17**), ibuprofen (**20**) or indomethacin (**22**), with O^2-chloromethyl compounds

95a or **132** using the polar aprotic solvent HMPA (Scheme 18). Once again, the use of HMPA proved to be more efficient than other solvents such as DMF, DMSO, THF or MeCN.


Aspirin, 17
(a)
95a or 132
89, 90
(95a)
Ibuprofen, 20
91, 92
Indomethacin, 22
93, 94
R =
89, 91, 93
90, 92, 94

Scheme 18: Reagents and conditions: a) Na_2CO_3, HMPA, 25 °C, 43 h.

In this regard, several generalizations describing the effect of solvents upon the rate of S_N^2 reactions have been widely described. Because hydrocarbons, halogenated hydrocarbons, and ethers are usually unsuitable solvents for reactions involving metal-ion salts, the reaction between sodium carboxylates obtained from aspirin, ibuprofen and indomethacin was carried out in polar aprotic solvents. Acetonitrile is somewhat more polar, but the solubility of the reactants in this solvent was low. The use of dimethylformamide (DMF), dimethylsulfoxide (DMSO), and hexamethylphosphoramide (HMPA) which are good solvents for salts, improved the yield of these reactions by virtue of selective cation solvation. Anionic nucleophiles usually show enhanced nucleophilicity in these solvents due to a reduction in the free energy difference between reactants and transition states (reduction in the activation anergy).

4.1.4.1. Experimental.

O^2-Sodium 1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate (130).

Dimethylamine (**129**, 4.5 g, 0.10 mol) was added to a solution of sodium methoxide (0.1 mol, 24 mL of a 25% w/v solution in methanol) and diethyl ether (300 mL) with stirring at 25 °C. This mixture was flushed with dry nitrogen for five minutes and then the reaction was allowed to proceed under an atmosphere of nitric oxide (40 psi internal pressure) with stirring at 25 °C for 19 h. The product, which precipitated as a fine white powder, was isolated by filtration and then suspended in diethyl ether (100 mL) upon stirring for 15 min. The suspension was filtered, and the solid collected was dried at 25 °C under reduced pressure until a constant weight was achieved after about 2 h to afford **130** as a fine white powder (11.5 g, 90 %); mp 258-260 °C (dec.); ^{1}H NMR (DMSO-d_6) δ 2.97 [s, 6H, N(CH_3)$_2$]. Product **130** was used immediately after drying without further purification for the preparation of compound **131**.

O^2-(Methylthiomethyl)-1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate (131).

The sodium diazeniumdiolate **130** (7.0 g, 55 mmol) was added to a suspension of potassium carbonate (1.5 g, 11 mmol) and HMPA (80 mL) at 4 °C and this mixture was stirred for 30 min. Chloromethyl methyl sulfide (6.3 g, 65.6 mmol) was added drop wise, and the reaction was allowed to proceed at 25 °C for 72 h with stirring. Ethyl acetate (200 mL) was added to quench the reaction, the solids were filtered off and the organic phase was washed with water (5 x 80 mL), dried (Na_2SO_4), and solvent was removed *in vacuo* to give a liquid residue which was purified by silica gel column chromatography using EtOAc-hexane (1:4, v/v) as eluent. Compound **131** (1.97 g, 21%) was obtained as a pale yellow liquid; ^{1}H NMR ($CDCl_3$) δ 2.24 (s, 3H, SC*H*$_3$), 3.01 [s, 6H,

$N(CH_3)_2]$, 5.21 (s, 2H, OCH_2S). Compound **131** was used immediately for the subsequent preparation of O^2-chloromethyl derivative **132**.

O^2-(Chloromethyl)-1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate (132).

A solution of compound **131** (1.8 g, 11 mmol) in dichloromethane (20 mL) was cooled to 4 °C, sulfuryl chloride (2.3 g, 17 mmol, 17 mL of a 1.0 M solution in dichloromethane) was added dropwise, the ice bath was removed and reaction mixture was stirred at 25 °C for 3 h. The brown solid suspended in the reaction media was removed by filtration and the solvent was evaporated to afford **132** (1.7 g, quantitative yield); ^{1}H NMR ($CDCl_3$) δ 3.01 [s, 6H, $N(CH_3)_2$], 5.76 (s, 2H, $ClCH_2O$). Compound **132** was used without further purification for the synthesis of products **90**, **92** and **94**.

General Method for the Preparation of •NO-NSAIDs (89-94).

Sodium carboxylates of the respective NSAID (aspirin, ibuprofen or indomethacin) were prepared *in situ* by stirring each acid (5 mmol) in a suspension of sodium carbonate (0.53 g, 5 mmol) and HMPA (7 mL) for 19 h at 25 °C. A solution of a O^2-(chloromethyl)diazen-1-ium-1,2-diolate **95a** or **132** (5 mmol) in HMPA (3 mL) was then added, and the reaction was allowed to proceed for 24 h at 25 °C. Ethyl acetate (60 mL) was added, the mixture was washed with water (5 × 30 mL), the organic phase was dried (Na_2SO_4), and the solvent was removed *in vacuo*. The residue obtained was purified by silica gel column chromatography using $CHCl_3$-EtOAc-hexane (35:15:50, v/v/v) as eluent for compounds **89**, **90**, **93**, and **94**; EtOAc-hexane (1:4, v/v) for compound **91**; and hexane-ether (3:1, v/v) for compound **92**. Physical and spectral data for **89-94** are listed below.

O^2-(Acetylsalicyloyloxymethyl)-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (89).

46 % yield; white crystals; mp 110-112 °C; IR ($CHCl_3$) 3019 (C-H arom), 2992 (C-H aliph), 1770 (CO_2), 1736 (CO_2), 1259, 1199 (N=N-O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.95 (quintet, J = 6.9 Hz, 4H, pyrrolidin-1-yl H-3, H-4), 2.34 (s, 3H, acetyl CH_3), 3.57 (t, J = 6.9 Hz, 4H, pyrrolidin-1-yl H-2, H-5), 5.97 (s, 2H, OCH_2O), 7.12 (d, J = 8.1 Hz, 1H, phenyl H-3), 7.34 (t, J = 8.1 Hz, 1H, phenyl H-5), 7.60 (td, J = 8.1, 1.5 Hz, 1H, phenyl H-4), 8.08 (dd, J = 8.1, 1.5 Hz, 1H, phenyl H-6). Anal. calcd. for $C_{14}H_{17}N_3O_6$: C, 52.01; H, 5.30; N, 13.00. Found: C, 51.99; H, 5.28; N, 12.90.

O^2-(Acetylsalicyloyloxymethyl)-1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate (90).

40 % yield; white crystals; mp 88-89 °C; IR (KBr) 3019 (C-H arom), 2979 (C-H aliph), 1756 (CO_2), 1609 (CO_2), 1219, 1184 (N=N-O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.34 (s, 3H, acetyl CH_3), 3.07 (s, 6H, $N(CH_3)_2$), 6.02 (s, 2H, OCH_2O), 7.12 (d, J = 8.1 Hz, 1H, phenyl H-3), 7.34 (t, J = 8.1 Hz, 1H, phenyl H-5), 7.60 (td, J = 8.1, 1.5 Hz, 1H, phenyl H-4), 8.07 (dd, J = 8.1, 1.5 Hz, 1H, phenyl H-6). Anal. calcd. for $C_{12}H_{15}N_3O_6$: C, 48.48; H, 5.09; N, 14.14. Found: C, 48.78; H, 4.97; N, 14.01.

O^2-[2-(4-(Isobutyl)phenyl)propanoyloxymethyl]-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (91).

58 % yield; yellow oil; IR (KBr) 2985 (C-H arom), 2864 (C-H aliph), 1750 (CO_2), 1286, 1129 (N=N-O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.89 [d, J = 6.6 Hz, 6H, $CH(CH_3)_2$], 1.50 (d, J = 7.2 Hz, 3H, $PhCHCH_3$), 1.79-1.89 [m, 1H, $CH(CH_3)_2$], 1.91-1.94 (m, 4H, pyrrolidin-1-yl H-3, H-4), 2.43 (d, J = 7.2 Hz, 2H, $PhCH_2CH$), 3.45-3.50 (m, 4H, pyrrolidin-1-yl H-2, H-5), 3.73 (q, J = 7.2 Hz, 1H, $PhCHCH_3$), 5.71 (d, J = 7.2 Hz, 1H, OCHH'O), 5.77 (d, J = 7.2 Hz, 1H, OCH'HO), 7.07 (d, J = 7.8 Hz, 2H, phenyl H-3, H-5), 7.19 (d, J = 7.8 Hz, 2H, phenyl H-2, H6). Anal.

calcd. for $C_{18}H_{27}N_3O_4$: C, 61.87; H, 7.79; N, 12.03. Found: C, 61.83; H, 7.79; N, 12.03.

O^2-[2-(4-(Isobutyl)phenyl)propanoyloxymethyl]-1-(*N*,*N*-dimethylamino) diazen-1-ium-1,2-diolate (92).

81 % yield; yellow oil; IR (KBr) 2959 (C-H arom), 2871 (C-H aliph), 1763 (CO_2), 1279, 1138 (N=N-O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.89 [d, *J* = 6.9 Hz, 6H, CH(C*H*$_3$)$_2$], 1.50 (d, *J* = 6.9 Hz, 3H, PhCH(C*H*$_3$), 1.83 [septet, *J* = 6.9 Hz, 1H, C*H*(CH$_3$)$_2$], 2.43 (d, *J* = 6.9 Hz, 2H, PhC*H*$_2$CH), 2.97 [s, 6H, N(C*H*$_3$)$_2$], 3.74 (q, *J* = 6.9 Hz, 1H, PhC*H*CH$_3$), 5.74 (d, *J* = 7.2 Hz, 1H, OC*H*'HO), 5.79 (d, *J* = 7.2 Hz, 1H, OCH'*H*O), 7.08 (d, *J* = 7.8 Hz, 2H, phenyl H-3, H-5), 7.19 (d, *J* = 7.8 Hz, 2H, phenyl H-2, H-6). Anal. calcd. for $C_{16}H_{25}N_3O_4$: C, 59.42; H, 7.79; N, 12.99. Found: C, 59.41; H, 7.80; N, 12.89.

O^2-[2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl) acetoxymethyl]-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (93).

51 % yield; yellow oil; IR (KBr) 3019 (C-H arom), 2979, 2885 (C-H aliph), 1756 (CON), 1689 (CO_2), 1293, 1165 (N=N-O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.88 (quintet, *J* = 6.9 Hz, 4H, pyrrolidin-1-yl H-3, H-4), 2.36 (s, 3H, C*H*$_3$), 3.40 (t, *J* = 6.9 Hz, 4H, pyrrolidin-1-yl H-2, H-5), 3.71 (s, 2H, C*H*$_2$CO$_2$), 3.83 (s, 3H, OC*H*$_3$), 5.77 (s, 2H, OC*H*$_2$O), 6.66 (dd, *J* = 9.0, 2.4 Hz, 1H, indol-3-yl H-6), 6.90 (d, *J* = 9 Hz, 1H, indol-3-yl H-7), 6.94 (d, *J* = 2.4 Hz, 1H, indol-3-yl H-4), 7.47 (d, *J* = 8.7 Hz, 2H, benzoyl H-3, H-5), 7.65 (d, *J* = 8.7 Hz, 2H, benzoyl H-2, H-6). Anal. calcd. for $C_{24}H_{25}ClN_4O_6$: C, 57.54; H, 5.03; N, 11.18. Found: C, 57.53; H, 5.03; N, 11.22.

O^2-[2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl) acetoxymethyl]-1-(N,N-dimethylamino)diazen-1-ium-1,2-diolate (94).

69 % yield; yellow oil; IR (KBr) 2979, 2925 (C-H aliph), 1763 (CON), 1689 (CO_2), 1333, 1064 (N=N-O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.35 (s, 3H, CH_3), 2.94 [s, 6H, $N(CH_3)_2$], 3.71 (s, 2H, CH_2CO_2), 3.81 (s, 3H, OCH_3), 5.80 (s, 2H, OCH_2O), 6.66 (dd, *J* = 8.7, 2.4 Hz, 1H, indol-3-yl H-6), 6.88 (d, *J* = 8.7 Hz, 1H, indol-3-yl H-7), 6.93 (d, *J* = 2.4 Hz, 1H, indol-3-yl H-4), 7.46 (d, *J* = 8.4 Hz, 2H, benzoyl H-3, H-5), 7.64 (d, *J* = 8.4, 2H, benzoyl H-2, H-6). Anal. calcd. for $C_{22}H_{23}ClN_4O_6$: C, 55.64; H, 4.88; N, 11.80. Found: C, 55.63; H, 4.89; N, 11.79.

4.2. Pharmacology.

4.2.1. Project 1: CC modulators possessing a O^2-alkyl-*N*-diazeniumdiolate moiety.

4.2.1.1. Preamble.

The 1,4-dihydropyridine group of CC modulators was of interest since the parent ring substituents can be manipulated to produce agonist and/or antagonist effects. In particular, potential use of 1,4-dihydropyridine CC agonists as positive inotropic agents to treat CHF warrants further investigation. 1,4-Dihydropyridine CC modulators actions are more specific for the L-type Ca^{2+} channels than other classes of CC modulators such as phenylalkylamines or the benzothiazepines. In comparison to the other classes of CC modulators, 1,4-dihydropyridines do not adversely affect other physiological systems outside of the cardiovascular system.

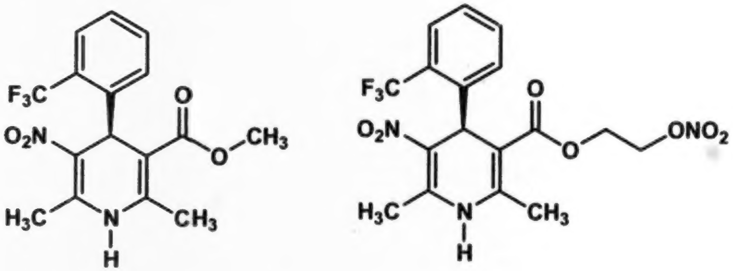
One of the major drawbacks of 1,4-DHP CC agonists for the potential treatment of CHF is their lack of cardioselectivity.[53] In order to abolish the smooth muscle contraction action of racemic CC agonists, a •NO donor group (diazeniumdiolate) was covalently attached to a 1,4-dihydropyridine moiety via an ester group. The addition of a diazeniumdiolate moiety to the parent compound may counter-balance the vascular constriction effect induced by CC agonists. Consequently, a project to design 1,4-dihydropyridine hybrid ester prodrugs that can simultaneously release •NO was initiated. It has been shown that nitrendipine facilitates the release of •NO from vascular endothelium which may contribute to its vascular relaxation effect,[336] that •NO modulates the activity of the calcium ion release channel by preventing oxidation of regulatory sulfhydryls,[337] and that •NO donors evoke a small but constant positive inotropic effect *in vivo* that is not caused by coronary vasodilation.[338]

1,4-Dihydropyridine CC modulators having different C-3 and C-5 ester substituents, are chiral molecules in which the chiral centre is located at the C-4 position. In 1,4-DHP CC modulators possessing a C-3 nitro substituent, one enantiomer usually acts as a CC agonist while the other acts as a CC antagonist.[62,107,339] Because both the agonist and antagonist effects play potentially beneficial roles in treating heart failure, isolation of the enantiomers from the racemate is often not necessary in preliminary studies.

For our CC agonist studies, a 4-benzofurazanyl or 2-trifluoromethylphenyl substituent was attached to the C-4 position based on the activity of the classical 1,4-dihydropyridine CC agonists BAY K 8644 (**11**) and PN 202-791 (**12**), respectively (Figure 5 on page 11), which are the two most potent 1,4-dihydropyridine CCags reported to date. BAY K 8644 and PN 202 791 possess a nitro substituent at the C-3 position. For this reason, the 1,4-dihydropyridine CCags described herein also possess a C-3 nitro moiety. All of the compounds synthesized have methyl substituents at both the C-2 and C-6 positions, since these substituents are reported to confer good CC modulating effects. An unsubstitued *N*-1 nitrogen was maintained because the proton attached to N-1, which acts as hydrogen bond donor in binding to CC receptor, is essential for calcium channel modulation activity.[62,69,104,340] The attachment of an aminoalkyl ester substituent at the C-5 position of the 1,4-dihydropyridine moiety, to mimic the slow onset and long duration of CC modulation effects, like amlodipine, was also considered but not investigated.[341]

Pharmacological evaluation of these types of CC modulators as CCags and CCants on both heart and smooth muscle would provide useful structure-activity relationships with respect to CC modulation and tissue specificity.

The discovery that organic nitrovasodilators such as nitroglycerin (**38**) exhibit their *in vivo* effect by circumventing the •NO-production system in the endothelium, to deliver •NO directly to arterial muscle cells, stimulated our studies to investigate hybrid Hantzsch-type 1,4-DHP CC antagonist / •NO donor agents.[74] In an earlier study in the Knaus program, a hybrid drug design technique was exploited to effectively abolish the adverse CC agonist effect of (–)-(*S*)-Bay K 8644 (**133**) on smooth muscle. In this regard, replacement of the ester methyl group of (–)-(*S*)-**133** by a 2-nitrooxyethyl ($O_2NOCH_2CH_2$) moiety afforded the (–)-(*S*)-2-nitrooxyethyl analog (**134**) which acted as a *dual cardioselective CC agonist / smooth muscle selective CC antagonist,* and as a •NO donor in the presence, or absence, of *N*-acetylcysteamine.[26]



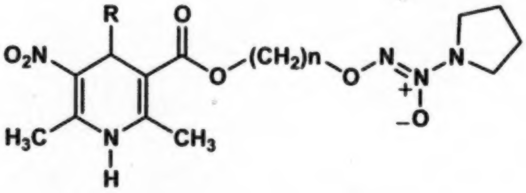
(**133**) (**134**)

Figure 33: chemical structures of (–)-(*S*)-BAY K 8644 (**133**) and the 2-nitrooxyethyl derivative (**134**).

Because diazeniumdiolate anions and their O^2-alkylation products have received considerable attention for use as •NO donor agents for biomedical research applications,[227] it was decided to use this •NO donor moiety as part of our on-going program to design hybrid CC modulation / •NO donor agents to treat CHF.

4.2.1.2. Results and discussion.

The CC agonist/antagonist modulation effects, and •NO-release studies for a group of O^2-alkyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate 1,4-dihydro-2,6-dimethyl-3-nitro-4-(benzofurazan-4-yl)pyridine-5-carboxylates (**67-70**), which constitutes this thesis work will be discussed in the context of a larger research work including the related analogues O^2-alkyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate 1,4-dihydro-2,6-dimethyl-3-nitro-4-[2-(trifluoromethyl)phenyl, 2-pyridyl, 3-pyridyl, and 4-pyridyl] pyridine-5-carboxylates **135-138** (Figure 34) which were synthesized by Dean Vo as part of the same project.[342]



135, R = 2-F_3C-C_6H_4-
136, R = 2-pyridyl
137, R = 3-pyridyl
138, R = 4-pyridyl

"a" series: n = 1
"b" series: n = 2
"c" series: n = 3
"d" series: n = 4

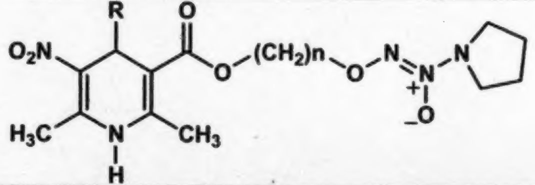
Figure 34: Additional 1,4-dihydropyridines possessing a C-5 O^2-(alkyl)-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate moiety.

Compounds having a C-4 3-pyridyl (**137b**, **137d**), or 4-pyridyl (**138b**, **138d**), substituent exhibited weak calcium channel antagonist activity (IC_{50} > 29.91 μM) on GPILSM, whereas compounds having a C-4 2-trifluoromethylphenyl (**135a**, **135c**, and **135d** with the exception of **135b**), 2-pyridyl (**136a-d**), or benzofurazan-4-yl (**67-70**), substituent exhibited moderate calcium channel antagonist activity (IC_{50}'s in the 0.55 to 38.6 μM range), relative to the reference drug nifedipine (IC_{50} = 0.014 μM). These data are listed in Table 3.

Some members (**135a-d**, **136c**, **138b**, **138d**, **67** and **70**) of the parent group of compounds retained their calcium channel cardiac agonist (positive inotropic) effect on GPLA (EC_{50}'s in the 0.096 to 44.66 μM range), relative to the reference drug Bay K 8644 (EC_{50} = 0.77 μM). The most potent cardiac calcium channel agonist, O^2-ethyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate

(**135a**) exhibited a potent positive inotropic effect (EC_{50} = 0.096 μM) that was about 8-fold more potent than that of Bay K 8644.

Table 2: In vitro calcium channel modulation activities for O^2-Alkyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate 4-aryl(heteroaryl)-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylates (**67-70**, and **135-138**).



Compd.	**n**	**R**	**Smooth muscle modulation**		**Cardiac Inotropy**
			GPILSM: IC_{50} (μM)[a]	**GPILSM: EC_{50} (μM)[b]**	**GPLA: EC_{50} (μM)[c]**
67	1	benzofurazan-4-yl	8.46 ± 0.95	No effect[d]	6.25 ± 1.88
68	2	benzofurazan-4-yl	0.55 ± 0.01	No effect[d]	No effect[e]
69	3	benzofurazan-4-yl	2.36 ± 0.15	No effect[d]	No effect[e]
70	4	benzofurazan-4-yl	0.96 ± 0.09	No effect[d]	2.45 ± 1.15
135a	1	2-(CF_3)phenyl	3.44 ± 0.13	4.89 ± 0.32	0.34 ± 0.15
135b	2	2-(CF_3)phenyl	69.51 (30.8 ± 5.9%)	No effect[d]	0.096 ± 0.01
135c	3	2-(CF_3)phenyl	38.6 ± 17.5	No effect[d]	1.28 ± 0.04
135d	4	2-(CF_3)phenyl	7.34 ± 1.04	No effect[d]	14.6 ± 9.3
136a	1	2-pyridyl	4.77 ± 0.10	No effect[d]	No effect[e]
136b	2	2-pyridyl	2.33 ± 0.36	No effect[d]	No effect[e]
136c	3	2-pyridyl	13.6 ± 0.08	No effect[d]	28.2 ± 5.4
136d	4	2-pyridyl	9.28 ± 0.77	No effect[d]	No effect[e]
137b	2	3-pyridyl	29.91 (15.1 ± 5.0%)	No effect[d]	No effect[e]
137d	4	3-pyridyl	29.91 (28.22 ± 10.0%)	No effect[d]	No effect[e]
138b	2	4-pyridyl	29.91 (22.84 ± 5.5%)	No effect[d]	44.66 (16.23 ± 11%)
138d	4	4-pyridyl	29.91	No effect[d]	7.05 ± 4.33

		(29.37 ± 5.1%)		
139[f]	—	4.87 ± 2.06	—	9.67 ± 0.59
140[f]	—	—	48	28.5 ± 2.0
141[f]	—	—	35	8.05 ± 2.14
nifedipine[f]	—	0.0143 ± 0.0038	—	—
BAY K 8644[f]	—	—	0.23 ± 0.01	0.77 ± 0.59
PN 202-791	—	0.04 ± 0.007	—	9.40 ± 2.6

[a] The micromolar concentration of the test compound causing a 50% decrease in the slow component or tonic contractile response (IC_{50} ± SEM, n = 3) in guinea pig ileum longitudinal smooth muscle (GPILSM) induced by the muscarinic agonist carbachol (0.167 μM) was determined graphically from the dose-response curves. For compounds **135b**, **137b**, **137d**, **138b**, and **138d** where an IC_{50} value could not be determined, the % decrease in the contractile response is listed in parentheses at the highest test compound concentration employed.

[b] The micromolar concentration of the test compound causing a 50% increase in the slow component or tonic contractile response (EC_{50} ± SEM, n=3) in guinea pig ileum longitudinal smooth muscle (GPILSM), in the absence of carbachol, was determined graphically from the dose-response curves.

[c] The micromolar concentration of the test compound causing a 50% increase in the cardiac contractile force (EC_{50} ± SEM, n=3) in guinea pig left atrium (GPLA) was determined graphically from the dose-response curves. For compound **138b**, where an EC_{50} value could not be determined, the % increase in contractile force at the highest test compound concentration employed is listed in parentheses.

[d] No smooth muscle calcium channel agonist response was observed at the highest test compound concentration employed (44.66 μM).

[e] No calcium channel agonist response (positive inotropic effect) on heart was observed at the highest test compound concentration employed (44.66 μM).

[f] Data for racemates are taken from the literature.[321]

In vitro smooth muscle CC antagonist activities for compounds **67-70** and **135-138** were determined using a reported GPILSM assay.[22] The micromolar concentration of the antagonist compound required to produce 50% inhibition of GPILSM Ca^{2+}-dependent contractility (IC_{50}) induced by the muscarinic agonist carbachol (0.167 μM) are presented in Table 3. With the exception of compound **135b** (IC_{50} > 69 μM), compounds having a C-4 2-trifluoromethylphen[illegible] (**135**), 2-pyridyl (**136**), or benzofurazan-4-yl (**67-70**) substituent exhibited more potent calcium channel antagonist activity (IC_{50}'s in the 0.55 to 38.6 μM range), than related analogs having a C-4 3-pyridyl (**137**), or 4-pyridyl (**138**) substituent with IC_{50} values > 29.91 μM, relative to the reference drug nifedipine (IC_{50} = 0.0143 μM). The point of attachment of C-4 isomeric pyridyl substituents was a determinant of antagonist activity where the relative potency profile was 2-pyridyl (**136a-d**, IC_{50} values in the 2.33 to 13.6 μM range) > 3-pyridyl (**137b**, **137d**) and 4-pyridyl (**138b**, **138d**) with IC_{50} values > 29.91 μM. Subgroups of compounds **135a-d**, **136a-d**, and **67-70** having alkyl spacer groups of variable chain length between the C-5 ester group and the O^2-diazeniumdiolate [$-CO_2(CH_2)_nO-$, n = 1-4] exhibited small differences in potency, indicating that the length of the alkyl spacer $(CH_2)_n$ was not a major determinant of calcium channel antagonist activity.

The *in vitro* effect of compounds **67-70**, and **135-138** on GPILSM in the absence of carbachol was also determined since racemic Bay K8644 (**11**) increases GPILSM contractility (EC_{50} = 0.23 μM) in the absence of carbachol. In this assay, the contractile response (EC_{50} value obtained from a dose-response curve) induced by the test compound on GPILSM in the absence of carbachol is compared to the reference compound carbachol, which is defined as producing a 100% increase in contractile force on GPILSM at a 0.167 μM concentration. The results from this study showed that replacement of the methyl ester substituent of racemic Bay K 8644 (**11**) by a 5-[O^2-alkyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate] ester moiety constitutes a suitable strategy to abolish the undesirable contractile agonist effect (vasoconstriction) of **11** on

GPILSM in the case of compounds **135b**, **135c**, and **135d**. By comparison, the contractile effect for **135a** (EC_{50} = 4.89 μM) was reduced about 21-fold relative to that observed for Bay K 8644 (0.23 μM). Compounds having other C-4 substituents such as **136** (R = 2-pyridyl), **137** (R = 3-pyridyl), **138** (R = 4-pyridyl) or **67-70** (R = benzofurazan-4-yl) had no effect on the contractile response of GPILSM in this latter assay.

In a previous study, Vo *et al.* reported that the racemic C-4 2-pyridyl (**139**), 3-pyridyl (**140**), and 4-pyridyl (**141**) compounds produced a cardiac calcium channel agonist action (positive inotropes) in an *in vitro* cardiac (GPLA) assay.[22]

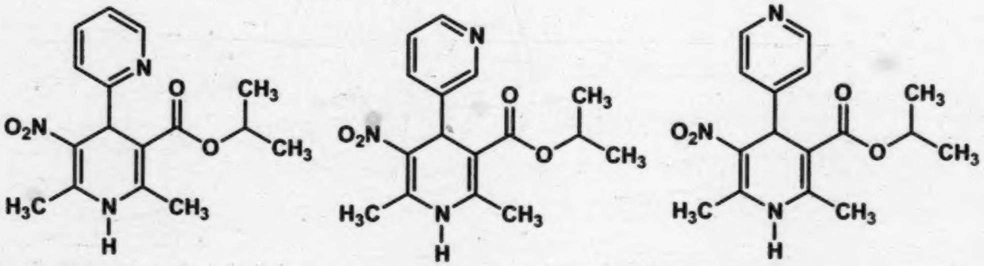

(139) **(140)** **(141)**

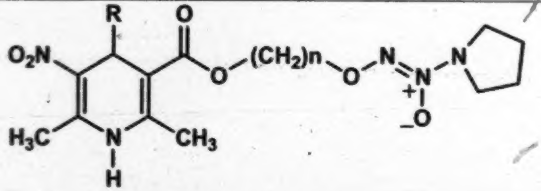
Figure 35: Racemic 1,4-dihydropyridine isomers previously reported as CC agonists in an *in vitro* GPLA assay.

The *in vitro* CC agonist activities (positive inotropic effect) for related compounds wherein the ester "methyl" moiety of Bay K 8644 (**11**), the ester "isopropyl" moiety of PN 202-791 (**12**) and the pyridyl isomers **139**, **140** and **141**, were replaced by an O^2-alkyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate group have now been determined (see data in Table 3). This replacement provided the Bay K 8644 group of analogs (**135a-d**) that retained the desired cardiac positive inotropic effect. The most potent compound in this group, O^2-ethyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate 1,4-dihydro-2,6-dimethyl-3-nitro-

4-(2-trifluoromethylphenyl)pyridine-5-carboxylate (**135b**, EC_{50} = 0.096 μM) is about a 8-fold more potent CC agonist than the reference compound Bay K 8644 (EC_{50} = 0.77 μM). Related compounds having a C-4 2-pyridyl (**136a-d**), 3-pyridyl (**137b**, **137d**), or 4-pyridyl (**138b**), ring system were generally less potent cardiac positive inotropes (EC_{50} values ≥ 28.2 μM) than the respective C-5 isopropyl parent compounds **139**, **140** or **141**. A similar replacement of the ester "isopropyl" group in the C-4 benzofurazan-4-yl group of compounds by an O^2-alkyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate ester substituent provided compounds **67** (n = 1, EC_{50} = 6.25 μM), or **70** (n = 4, EC_{50} = 2.45 μM) that were approximately equipotent positive inotropes with the parent reference compound PN 202-791 (**12**, EC_{50} = 9.40 μM). In contrast, related analogs **68** (n = 2), and **69** (n = 3) were less active (EC_{50} values > 44.66 μM). The observed differences in positive inotropic activities for compounds **67-70**, **135-138** could be attributable to a number of possibilities such as differences in the drug-receptor interaction, and/or preferential affinity for, or access to, the resting (R), open (O), or inactivated (I) states of the L-type calcium channel receptor.[343]

The *in vitro* release of •NO from this group of compounds (**67-70**, **135-138**) in the presence of either L-cysteine, pig liver esterase, or rat serum, during a 1.5 h incubation at 37°C was quantified as nitrite (NO_2^-) produced by the reaction of nitric oxide with oxygen and water using the Griess reagent. The mol/mol % of nitric oxide released (< 1%) by this group of compounds was approximately 110-fold lower than that for the reference compound (59%) O^2-sodium 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (**57**). These data are listed in Table 4.

Table 2: Nitric oxide release studies for *O*^{2}-alkyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate 4-aryl(heteroaryl)-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylates (**67-70, 135-138**).



Compd.	n	R	Percent nitric oxide released[a]		
			PBS L-cysteine[b]	PBS with esterase[c]	PBS with rat serum[d]
67	1	benzofurazan-4-yl	0.39 ± 0.05	0.52 ± 0.01	0.62 ± 0.03
68	2	benzofurazan-4-yl	0.42 ± 0.04	0.58 ± 0.05	0.63 ± 0.02
69	3	benzofurazan-4-yl	0.51 ± 0.02	0.55 ± 0.03	0.63 ± 0.03
70	4	benzofurazan-4-yl	0.48 ± 0.02	0.52 ± 0.05	0.62 ± 0.03
135a	1	2-(CF_3)phenyl	0.45 ± 0.05	0.52 ± 0.02	0.62 ± 0.03
135b	2	2-(CF_3)phenyl	0.40 ± 0.02	0.59 ± 0.03	0.60 ± 0.01
135c	3	2-(CF_3)phenyl	0.49 ± 0.03	0.50 ± 0.06	0.64 ± 0.02
135d	4	2-(CF_3)phenyl	0.32 ± 0.07	0.54 ± 0.03	0.64 ± 0.04
136a	1	2-pyridyl	0.50 ± 0.01	0.52 ± 0.04	0.58 ± 0.03
1336b	2	2-pyridyl	0.38 ± 0.06	0.48 ± 0.03	0.50 ± 0.05
1336c	3	2-pyridyl	0.45 ± 0.02	0.56 ± 0.03	0.60 ± 0.03
1336d	4	2-pyridyl	0.36 ± 0.04	0.49 ± 0.02	0.56 ± 0.02
137a	1	3-pyridyl	0.45 ± 0.02	0.55 ± 0.01	0.65 ± 0.01
137b	2	3-pyridyl	0.48 ± 0.04	0.55 ± 0.04	0.65 ± 0.04
137c	3	3-pyridyl	0.40 ± 0.03	0.42 ± 0.05	0.53 ± 0.05
137d	4	3-pyridyl	0.52 ± 0.05	0.60 ± 0.03	0.57 ± 0.03
138a	1	4-pyridyl	0.52 ± 0.03	0.56 ± 0.06	0.59 ± 0.06
138b	2	4-pyridyl	0.49 ± 0.02	0.52 ± 0.04	0.55 ± 0.04
138c	3	4-pyridyl	0.50 ± 0.02	0.57 ± 0.02	0.64 ± 0.02
138d	4	4-pyridyl	0.46 ± 0.04	0.43 ± 0.01	0.57 ± 0.05

[a] Percent of nitric oxide released (± SEM, n=3) quantified as nitrite using the Griess reagent, relative to a theoretical maximum release of 2 mol of •NO/mol of drug.

[b] Incubated in the presence of 5 mM L-cysteine in phosphate buffer solution (pH 7.4) at 37 °C for 1.5 h.

[c] Incubated in the presence of 10 equivalents of pig liver esterase (based on a ratio 1 mol of test compound/10 mol of esterase) in phosphate buffer solution (pH 7.4) at 37 °C for 1.5 h.

[d] Incubated in the presence of 90 μL of rat serum in phosphate buffer solution (pH 7.4) at 37 °C for 1.5 h.

It has been reported that a reduced thiol such as L-cysteine, L-cysteamine or glutathione is required for the release of •NO from certain •NO donors such as the furoxan moiety.[344] However, the percent •NO released from the O^2-alkyldiazeniumdiolates esters **67-70, 135-138** was negligible (0.32 to 0.52% range) upon incubation with L-cysteine (either 3.4 or 5.0 mM), relative to the reference •NO donor compound sodium 1-(pyrrolidine-1-yl)diazen-1-ium-1,2-diolate (**57**, 59% release). These data suggest that acyclic O^2-alkylated diazeniumdiolate esters (**67-70, 135-138**), unlike the structurally related cyclic furoxan ring system, are much more stable towards nucleophilic reactions promoted by the reduced thiol (-SH) substituent present in L-cysteine.

It has been reported[240] that the enzymatic release of •NO from O^2-acetoxymethylated diazeniumdiolate derivatives of diethylamine and pyrrolidine, upon incubation with porcine liver esterase, resulted in a rapid disappearance of the O^2-alkylated prodrug (half-lives from 3 seconds up to 2 minutes) to afford the original secondary amine from which it was derived (diethyl amine or pyrrolidine), and 1.8 mol of •NO / mol of prodrug. It was therefore of interest to determine the effect of esterases such as pig liver esterase, and esterases present in rat plasma, on •NO release from compounds **67-70** and **135-138**, especially those having a methylene spacer (**67**, **135a**, **136a**, **137a**, **138a**, n = 1) between the DHP 5-carboxylate and the diazeniumdiolate O^2-atom. It was anticipated that enzymatic ester cleavage would give rise to the respective parent carboxylic acids (**96**, **106-109**), and 1 mol of the O^2-hydroxymethyl compound (**142**). Spontaneous fragmentation of compound **142**, by release of formaldehyde would give the 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (**57**) that can theoretically generate two moles of •NO and 1 mol of pyrrolidine as illustrated in Figure 36.

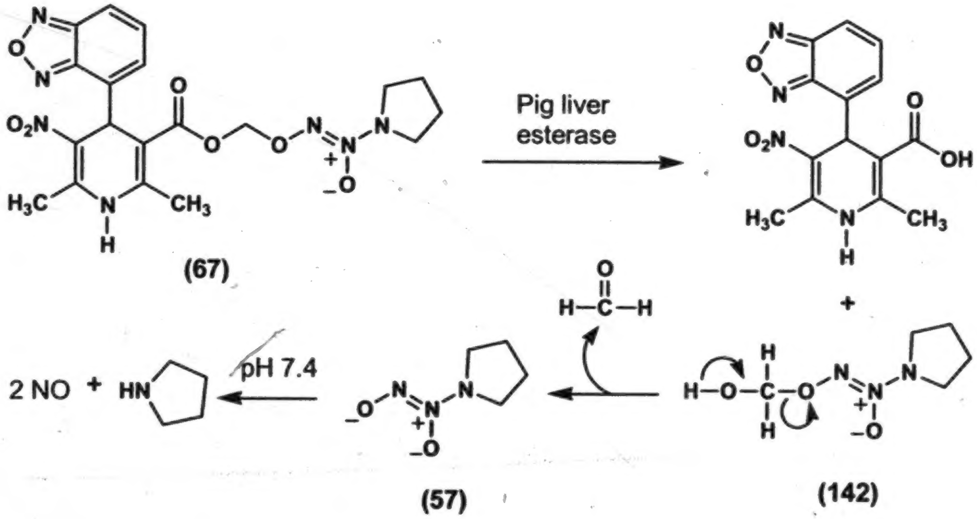


Figure 36: Theoretical ester cleavage and nitric oxide release from O^2-methyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate 4-aryl-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylates (**67** as an example).

Contrary to this expectation, the •NO release studies showed there was no difference in •NO release (0.42 to 0.65 percent range) upon incubation with either pig liver esterase, or rat plasma, between compounds having an O^2-methyl (**67**, **135a-138a**), O^2-ethyl (**68, 135b-138b**), O^2-propyl (**69, 135c-138c**), or O^2-butyl (**70, 135d-138d**) spacer group. These data indicate that O^2-alkyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate 4-aryl-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylates (**67-70**, **135-138**), unlike the O^2-acetoxymethylated diazeniumdiolate (**62**, Figure 21, page 45), are more stable towards enzymatic ester cleavage. One plausible explanation for the failure of esterases to cleave the ester moiety of compounds **67-70, 135-138** could be due to conjugation of the DHP-enamine 5,6-olefinic bond with the C-5 ester carbonyl group such that the bond between the DHP C-5 and the C-atom of the ester group has partial double-bond character making the ester more resistant to enzymatic cleavage by esterases. This explanation is consistent with the observation that the length of the alkyl spacer (n = 1 *versus* n = 2-4) in the O^2-alkyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate ester moiety for compounds **67-70, 135-138** was not a determinant of O^2-alkyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate ester •NO release.

The results from this study indicate that an O^2-alkyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate ester moiety is not a suitable •NO donor moiety for the design of Hantzsch 1,4-DHP hybrid cardioselective calcium channel agonist (positive inotropic) / smooth muscle selective calcium channel antagonist modulators in which the simultaneous release of the natural vasodilator •NO could counteract any calcium channel agonist effect (vasoconstriction) on vascular smooth muscle.

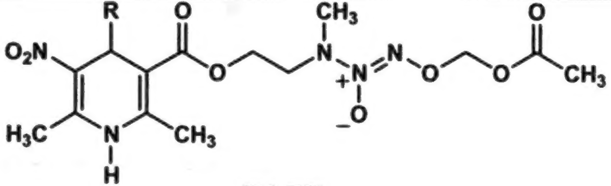
4.2.2. Project 2: CC modulators possessing a O^2-acetoxymethyl-*N*-diazeniumdiolate moiety.

A group of racemic 4-aryl(heteroaryl)-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylates possessing a potential nitric oxide donor O^2-acetoxymethyl-1-(*N*-ethyl-*N*-methylamino)diazen-1-ium-1,2-diolate (**71-75**), and O^2-acetoxymethyl-1-(4-ethylpiperazin-1-yl)diazen-1-ium-1,2-diolate (**76-80** see structures on next page), C-5 ester substituent were synthesized.

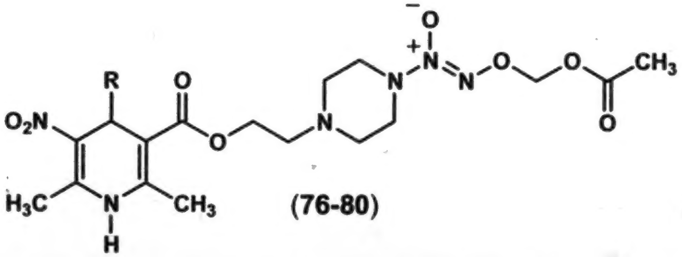
The point of attachment of C-4 isomeric pyridyl substituents was a determinant of CC antagonist activity on guinea pig ileum longitudinal smooth muscle (GPILSM), where the relative potency profile was 4-pyridyl > 2-pyridyl > 3-pyridyl. Compounds having a 2-pyridyl (**72**, **77**), 4-pyridyl (**74**, **79**), 2-trifluoromethylphenyl (**75**, **80**) and benzofurazan-4-yl (**71**, **76**) substituent at the 1,4-DHP C-4 position, exhibited more potent smooth muscle calcium channel antagonist activity (IC_{50}'s in the 0.37 to 1.09 μM range) than related analogs having a C-4 3-pyridyl substituent (**73** and **78**, IC_{50}'s = 9.14 and 3.03 μM), relative to the reference drug nifedipine (IC_{50} = 9.13 nM).

Replacement of the methyl ester moiety of Bay K 8644 by an O^2-acetoxymethyl-1-(*N*-ethyl-*N*-methylamino)diazen-1-ium-1,2-diolate group (**75**), or an O^2-acetoxymethyl-1-(4-ethylpiperazin-1-yl)diazen-1-ium-1,2-diolate group (**80**), retained the desired cardiac positive inotropic effect on guinea pig left atrium (GPLA, IC_{50}'s = 4.82 and 4.05 μM) although **75** and **80** were less potent than the reference drug Bay K 8644 (IC_{50} = 0.30 μM). In contrast to Bay K 8644, which exhibited a contraindicated CC agonist effect on GPILSM (EC_{50} = 0.23 μM), none of the compounds possessing a nitric oxide donor moiety (**71-80**) showed a CC agonist effect on GPILSM. Unlike analogs of PN 202-791 (**12**) having nitrooxyalkyl [$O_2NO(CH_2)n$] C-5 ester substituents reported in a recent study,[345] replacement of the isopropyl ester group of PN 202-791 by an O^2-acetoxymethyl-1-(*N*-ethyl-*N*-methylamino)diazen-1-ium-1,2-diolate group (**71**), or an O^2-acetoxymethyl-1-(4-ethylpiperazin-1-yl)diazen-1-ium-1,2-diolate group (**76**), decreased (**71**) or even abolished (**76**) the desired cardiac positive inotropic effect on GPLA. A complete list of the CC modulation results are presented in Table 5.

Table 3: *In vitro* calcium channel modulation activities for 4-aryl(heteroaryl)-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylates possessing a O^2-acetoxymethyl-1-(*N*-ethyl-*N*-methylamino)diazen-1-ium-1,2-diolate (**71-75**), or O^2-acetoxymethyl-1-(4-ethylpiperazin-1-yl)diazen-1-ium-1,2-diolate (**76-80**), C-5 ester substituent.



(71-75)



(76-80)

Compd.	R	GPILSM IC$_{50}$ (μM)[a]	GPILSM EC$_{50}$ (μM)[b]	GPLA EC$_{50}$ (μM)[c]
71	2-pyridyl	0.89 ± 0.01	No effect[d]	No effect[e]
72	3-pyridyl	9.14 ± 6.25	No effect[d]	No effect[e]
73	4-pyridyl	0.37 ± 0.03	No effect[d]	32.2 ± 3.2
74	2-(CF$_3$)phenyl	0.57 ± 0.13	No effect[d]	4.82 ± 1.41
75	benzofurazan-4-yl	0.46 ± 0.07	No effect[d]	22.7 ± 1.7
76	2-pyridyl	1.09 ± 0.02	No effect[d]	No effect[e]
77	3-pyridyl	3.03 ± 0.20	No effect[d]	8.18 ± 0.71
78	4-pyridyl	0.60 ± 0.05	No effect[d]	12.7 ± 6.7

79	2-(CF_3)phenyl	0.84 ± 0.01	No effect[d]	4.05 ± 0.23
80	benzofurazan-4-yl	0.97± 0.01	No effect[d]	No effect[e]
Nifedipine		0.0091 ± 0.0026	–	–
BAY K 8644		–	0.23 ± 0.01	0.30 ± 0.01
PN 202-791		–	–	9.4 ± 1.2

[a] The micromolar concentration of the test compound causing a 50% decrease in the slow component or tonic contractile response (IC_{50} ± SEM, n = 3) in guinea pig ileum longitudinal smooth muscle (GPILSM) induced by the muscarinic agonist carbachol (0.167 μM) was determined graphically from the dose-response curve.

[b] The micromolar concentration of the test compound causing a 50% increase in the slow component or tonic contractile response in guinea pig ileum longitudinal smooth muscle (GPILSM), in the absence of carbachol, was determined graphically from the dose-response curves.

[c] The micromolar concentration of the test compound causing a 50% increase in the cardiac contractile force (EC_{50} ± SEM, n = 3) in guinea pig left atrium (GPLA) was determined graphically from the dose-response curves.

[d] No smooth muscle calcium channel agonist response was observed at the highest test compound concentration employed (44.66 μM).

[e] No calcium channel agonist response (positive inotropic effect) on heart was observed at the highest test compound concentration employed (44.66 μM).

It has been reported that the rate of •NO release from *O*^{2}-sodium *N*-substituted-diazen-1-ium-1,2-diolate salts varies greatly depending on the nature of the structure of the substrate.[227] One type of chemical modification used to control the rate of nitric oxide release from diazen-1-ium-1,2-diolates is the attachment of alkyl substituents to the *O*^{2}-position.[224] *O*^{2}-substituted diazen-1-ium-1,2-diolates are stable compounds that hydrolyze slowly even in acidic solution. Consistent with these observations, when compounds **71-80** were incubated in PBS at pH 7.4, the percentage of •NO released varied from 6.3 to 9.4 % suggesting a slow decomposition even when the media was acidified by quenching with the Griess reagent (pH about 1-2).

As mentioned previously, the prodrug O^2-acetoxymethyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (**62**) was reported to release up to 1.8 mol of •NO per mol hydrolysis of the O^2-acetoxymethyl group by porcine liver esterase (PLE). The first enzymatic hydrolysis product of **62**, O^2-hydroxymethyl-1-(pyrrolidin-1yl)diazen-1-ium-1,2-diolate (**142**), is reported to be unstable in water as it spontaneously eliminates formaldehyde and the zwitterion 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate which in turn releases nitric oxide in phosphate buffer (see the mechanism in Figure 37). •NO release data acquired in this investigation indicated that the amount of •NO released upon incubation of the test compounds **71-80** in PBS at pH 7.4 for 1.5 hours at 37 °C was relatively constant (6.3-9.4% range).

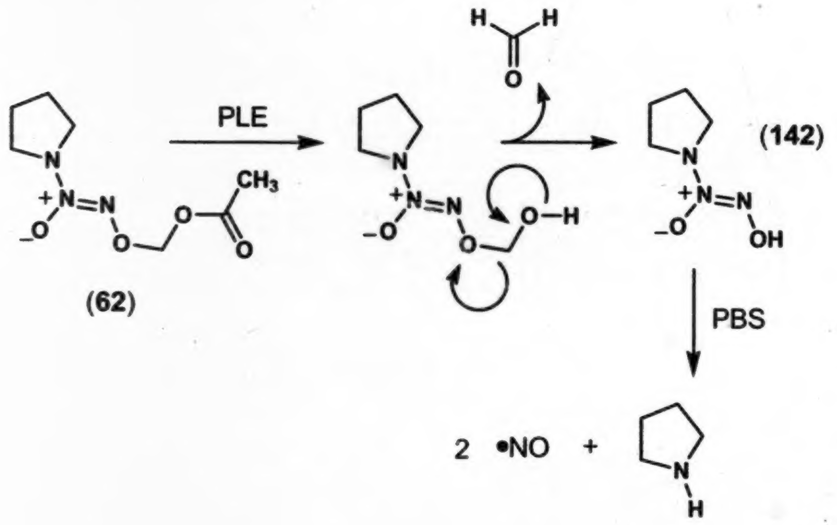


Figure 37: enzymatic hydrolysis of prodrug **62** in the presence of porcine liver esterase (PLE) and spontaneous •NO release from the O^2-hydroxymethyl-1-(pyrrolidin-1-yl)diazeniumdiolate (**142**).

The O^2-acetoxymethyl moiety of O^2-acetoxymethyl-1-(alkylamino)diazen-1-ium-1,2-diolate moieties, attached to 4-aryl(heteroaryl)-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylic acid derivatives, upon incubation with 10 equivalents of pig liver esterase (PLE) is hydrolyzed less extensively compared to when the O^2-acetoxymethyl moiety is present in smaller molecules such as the prodrug **62**. In this regard, incubation of compounds **71-80** in the presence of PLE produced a 21-34% release of •NO compared with the 90% •NO release from the prodrug **62**.

The effect of non-specific esterases on the •NO release properties of compounds **71-80** was determined by their incubation in the presence of guinea pig serum for 1.5 hours at 37 °C (pH 7.4). The percentage •NO released was substantially higher (60-75% range) than that observed upon incubation with PLE (see Table 6). These data indicate the non-specific serum esterases present in guinea pig serum cleave these O^2-acetoxymethyl-1-(alkylamino)diazen-1-ium-1,2-diolates more effectively than pig liver esterase (PLE). Compounds **71-80** are prodrugs which must be cleaved by esterases before they are able to release •NO. In contrast, the unprotected diazen-1-ium-1,2-diolates **113** and **118**, which lack the O^2-acetoxymethyl moiety and do not require cleavage by an esterase, released about 85% (**113**) and 70% (**118**) of the theoretical amount of •NO irrespective of whether the compound was incubated with PBS (pH 7.4), PLE, or guinea pig serum.

Table 4: Nitric oxide release studies for 4-aryl(heteroaryl)-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylates possessing a O^2-acetoxymethyl-1-(*N*-ethyl-*N*-methylamino)diazen-1-ium-1,2-diolate (**71-75**), or O^2-acetoxymethyl-1-(4-ethylpiperazin-1-yl)diazen-1-ium-1,2-diolate (**76-80**), C-5 ester substituent.

Compd.	**% of Nitric oxide released**[a]		
	PBS (pH 7.4)[b]	**PLE**[c]	**GP-Serum**[d]
71	9.42 ± 0.01	26.43 ± 0.06	68.19 ± 0.04
72	7.71 ± 0.04	23.48 ± 0.07	63.37 ± 0.05
73	9.03 ± 0.04	26.62 ± 0.05	62.41 ± 0.06
74	7.92 ± 0.05	23.95 ± 0.03	75.80 ± 0.04
75	6.32 ± 0.09	25.12 ± 0.06	69.84 ± 0.05
76	6.92 ± 0.05	34.90 ± 0.06	61.20 ± 0.04
77	6.93 ± 0.52	31.00 ± 0.05	—
78	7.81 ± 0.04	23.20 ± 0.05	60.73 ± 0.05
79	6.54 ± 0.06	21.85 ± 0.05	69.01 ± 0.06
80	7.84 ± 0.04	32.08 ± 0.05	72.53 ± 0.09
113	84.90 ± 0.06	84.92 ± 0.06	84.94 ± 0.05
118	70.45 ± 0.04	70.41 ± 0.05	70.46 ± 0.06

[a] Percent of nitric oxide released (± SEM, n = 3) quantified as nitrite using the Griess reaction, relative to a theoretical maximum release of 2 mol of •NO/mol of test compound.

[b] Incubated in phosphate buffer solution (PBS) only (pH 7.4) at 37 °C for 1.5 h.

[c] Incubated in the presence of 10 equivalents of pig liver esterase (based on a ratio of 1 mol of test compound / 10 mol of esterase) in phosphate buffer solution (pH 7.4) at 37 °C for 1.5 h.

[d] Test compound (2.0×10^{-4} mmol) incubated with guinea pig serum (260 µL) in phosphate buffer solution (pH 7.4) at 37 °C for 1.5 h.

The hybrid CC modulation/•NO donor drugs (**71-80**) possess a number of potential advantages, relative to using a physical mixture of a CC modulator and an organic nitrate vasodilator such as glycerol trinitrate:

i) the 1,4-DHP moiety can act as a carrier to simultaneously deliver the 1,4-DHP CC modulator and •NO moiety to the target tissue that could provide a synergistic effect. CC antagonists enhance the effect of •NO in vascular smooth muscle cells, the vasodilating effect induced by CC antagonists is increased by •NO-donor drugs,[346] and the combined effects of basal •NO release and CC antagonists produce an inhibition greater than additive where the concentrations of CC antagonist drug required (IC_{50}) is 3-fold lower in the presence of basal •NO release than in its absence.[347]

ii) In contrast to organic nitrate vasodilators, these hybrid compounds do not require a thiol cofactor as L-cysteine or glutathione to enhance the release of •NO from the diazen-1-ium-1,2-diolate moiety, and a redox activation is not needed for •NO release.[172]

iii) The rate of •NO release from the diazen-1-ium-1,2-diolate moiety can be controlled by the nature of the R-substituent in $R\text{-}N^+(O^-)\text{=}N\text{-}O^-$ compounds,[15,27] *and* compounds of general structure $R^1(R^2)N\text{-}N^+(O^-)\text{=}N\text{-}O(CH_2)nNH_2$ with $t_{1/2}$ values of 1.3 to 3400 minutes at 22 °C and pH 7.4 in phosphate buffer have been reported.[28]

iv) After release of •NO from the diazen-1-ium-1,2-diolate moiety at pH 7.4 (no enzyme required), the DHP released with a C-5 aminoalkyl ester substituent (**143**, Figure 38) is expected to have a long duration of action (like amlodipine which has an aminoethoxymethyl substituent) that may be amenable to once-a-day dosing which would circumvent the peak and trough plasma levels observed with short acting CC antagonist drugs such as nifedipine.

v) Hybrid compounds having an O^2-acetoxymethyldiazen-1-ium-1,2-diolate moiety may inhibit platelet aggregation like aspirin that is used chronically in low doses for prophylaxis of stroke and myocardial infarction,[266] and undergo rapid cleavage of the acetoxy group by plasma esterases prior to conversion to the diazen-1-ium-1,2-diolate •NO donor moiety which will subsequently release •NO (see Figure 38).

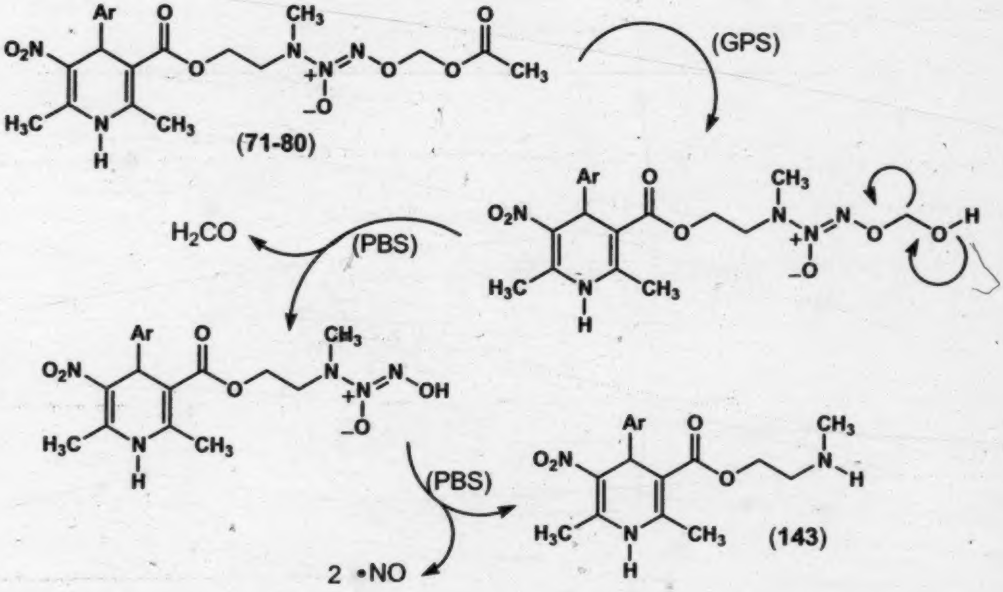

(71-80)
(GPS)
(PBS)
H_2CO
(PBS)
2 •NO
(143)

Figure 38: theoretical ester cleavage by guinea pig serum (GPS) of compounds **71-80** in phosphate buffer solution (PBS).

4.2.3. Project 3: 3,4-diphenylfuroxans (81-84) and 3,4-diphenylfurazans (85-88).

4.2.3.1. Preamble.

Despite the relatively safe pharmacological profile of selective COX-2 inhibitors, there is now increasing concern regarding their use in patients at risk for an adverse cardiovascular event such as myocardial infarction. This increased risk is thought to be triggered by a reduction in the level of the desirable platelet aggregation inhibitor and vasodilatory prostacyclin (PGI_2) in conjunction with an increased level of the undesirable potent platelet activator and aggregator thromboxane A_2 (TxA_2).[167] This biochemical explanation constitutes the rationale for the recent voluntary worldwide withdrawal of Vioxx® (rofecoxib, **23**). This decision was based on data, from a three-year clinical trial evaluating the ability of rofecoxib to reduce colon polyp recurrence, that revealed a "discernible and confirmed" higher risk of cardiovascular events such as stroke and heart attack in patients taking the drug for a period longer than eighteen months.[157] This latter clinical evidence presents a new challenge in the design of selective COX-2 inhibitors since it is now necessary to also consider the physiological consequences associated with alterations in the arachidonic acid cascade due to unbalanced inhibition of the COX-1 and COX-2 isozymes.

Hybrid molecules, comprised of a selective COX-2 inhibitor and •NO donor moieties, constitute one of the more promising approaches for the design of drugs which are devoid of the potential adverse cardiovascular effects associated with use of classical selective COX-2 inhibitors, and which elicit a decreased ulcerogenicity relative to that frequently observed on long-term use of traditional NSAIDs.[325] It has been reported that an increased generation of endothelial •NO, or release of •NO from a nitric oxide donor drug, is expected to produce beneficial effects such as a reduction in blood pressure and prevention of atherosclerosis.[179] At nanomolar concentrations, •NO reversibly activates soluble guanylate cyclase by 400-fold, catalyzing the conversion of guanosine triphosphate to cyclic guanosine monophosphate (cGMP, see Figure 13).[208] Elevation of cGMP relaxes smooth muscle in blood vessels, inhibits platelet aggregation and adhesion, and blocks the adhesion of white cells to blood vessel walls.[209] In addition to these cardiovascular effects, •NO is now recognized as a critical mediator of gastrointestinal mucosal defence, exerting many of the same actions as prostaglandins in the gastrointestinal tract.[325,348] Drugs classified as •NO-NSAIDs suppress COX-2 derived prostaglandin synthesis as effectively as the parent drugs, and they have been found to exert comparable antiinflammatory and antipyretic activity to the parent NSAID drug.[247,248]

1,2,5-Oxadiazol-2-oxides (furoxans) represent one class of heterocyclic compounds that are thiol-dependent •NO donor agents. In this context, furoxans are considered to be •NO releasing prodrugs whose biological activity is produced by action on the sGC-cGMP pathway. Compared to other •NO donor agents, furoxans possess rather favorable pharmacological properties, since they frequently release •NO slowly resulting in a longer duration of action. The absence of tolerance is an important distinctive feature of the furoxan moiety. A mechanism has been proposed by Granik and Grigor (see Scheme 3) for the thiol-induced release of •NO from furoxans that involves attack by a thiolate anion at C-3 and/or C-4 of the furoxan ring followed by ring opening and the subsequent release of •NO.[172]

4.2.3.2. Results and discussion.

As part of our ongoing research program to obtain selective COX-2 inhibitors with reduced cardiovascular toxicity, we investigated the synthesis, *in vitro* COX-1 / COX-2 inhibitory activities and •NO release data, for a group of 3,4-diphenyl-1,2,5-oxadiazole-2-oxides (**81-84**; 3,4-diphenylfuroxans) and the corresponding deoxy 3,4-diphenyl-1,2,5-oxadiazole (**85-88**; 3,4-diphenylfurazans) analogs, possessing a *para*-H, -SO_2CH_3, -SO_2NH_2 or -SO_2N_3 phenyl substituent, as potential hybrid COX-2 inhibitor / •NO donor drugs.

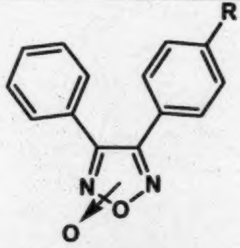
In vitro COX-1 and COX-2 enzyme inhibition data (Table 7) showed that replacement of the 2-(5*H*)furanone central ring present in rofecoxib (**23**) by a 1,2,5-oxadiazole-2-oxide ring maintains COX-2 inhibitory activity. Compounds **81** (COX-1 IC_{50} = 12.0 μM; COX-2 IC_{50} > 100 μM) and **85** (COX-1 IC_{50} = 0.15 μM; COX-2 IC_{50} > 100 μM), having unsubstituted C-3 and C-4 phenyl substituents, are selective COX-1 inhibitors. However, incorporation of a *para*-SO_2Me phenyl substituent (COX-2 pharmacophore) provided a mixture of the two regioisomers **82a** and **82b** [COX-1 IC_{50} = 11.6 μM; COX-2 IC_{50} = 0.12 μM; COX-2 selectivity index (S.I.) = 97], and **86** (COX-1 IC_{50} = 1.6 μM; COX-2 IC_{50} = 0.74 μM; COX-2 S.I. = 2), that possess a selectivity for the COX-2 isozyme. Compounds **83** and **87**, possessing a *para*-SO_2NH_2 phenyl substituent (COX-2 pharmacophore), like **82** and **86**, were also selective COX-2 inhibitors where the respective COX-2 selectivity indexes were 12 and 5, respectively.

In earlier investigations, we showed that 2-(5*H*)furanone rofecoxib analogs[349] possessing a *para*-SO_2N_3 phenyl substituent (**144** and **145**), and the *meta*-azidosulfonyl celecoxib analog[155] (**146,** Figure 39) exhibited significant COX-2 inhibitory activities. In contrast, in this study replacement of the SO_2Me substituent present in compounds **82** and **86**, or the SO_2NH_2 substituent present in compounds **83** and **87**, by a SO_2N_3 phenyl substituent provided compounds **84** and **88** which were completely devoid of both COX-1 and COX-2 inhibitory activity (COX-1 and COX-2 IC_{50} > 100 μM).

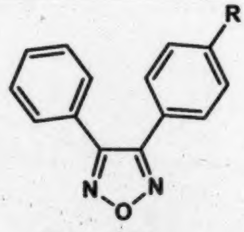
A comparison of the furoxan regioisomers (**82a-82b**) having a *N*-oxido substituent, with the corresponding furazan (**86**) that does not possess a *N*-oxido substituent, shows that *N*-deoxygenation provided a small increase in

COX-1 potency, and a modest decrease in COX-2 potency, which resulted in a lower COX-2 inhibitory selectivity index.

Table 5: *In vitro* COX inhibition data for 3,4-diphenylfuroxans (**81-84**) and 3,4-diphenylfurazans (**85-88**).



(81-84)

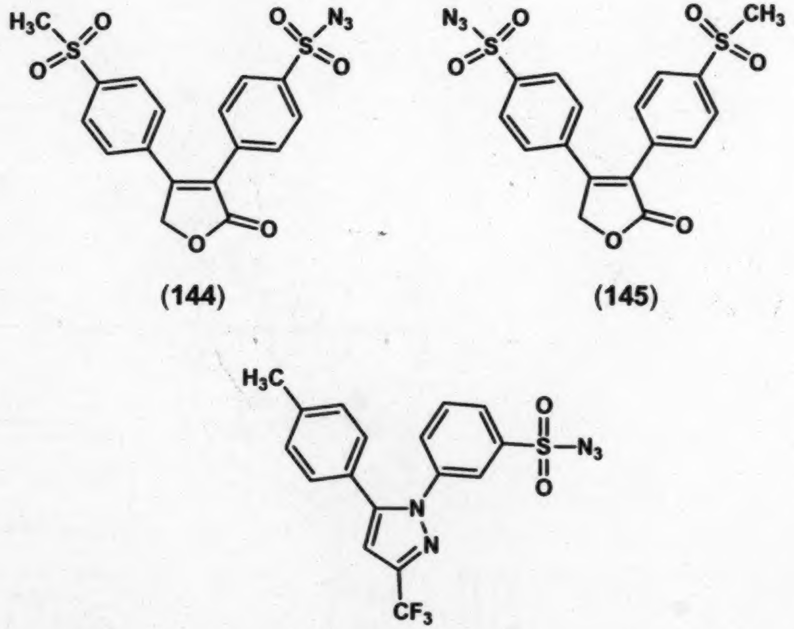


(85-88)

Compd.	COX-1 IC_{50} (μM)[a]	COX-2 IC_{50} (μM)[a]	COX-2 S.I.[b]
81	12.0	> 100	-
82a-82b	11.6	0.12	97
83	9.8	0.78	12
84	> 100	> 100	-
85	0.15	> 100	-
86	1.6	0.74	2
87	4.6	0.91	5
88	> 100	> 100	-
Rofecoxib	> 100	0.50	> 200
Celecoxib	33.1	0.07	472

[a] Values are means of two determinations acquired using an ovine COX-1/COX-2 assay kit (Catalog No. 560101, Cayman Chemicals Inc., Ann Arbor, MI, USA) and the deviation from the mean is <10% of the mean value.

[b] In vitro COX-2 selectivity index (COX-1/COX-2 IC_{50}).



(144) (145) (146)

Figure 39: previously reported azidosulfonyl analogs of rofecoxib (**144** and **145**) and celecoxib (**146**).

The percent •NO released from the 3,4-diphenyl-1,2,5-oxadiazole-2-oxides (furoxans **81-84**) upon *in vitro* incubation with L-cysteine, for 1.5 hours at 37°C, was determined and the results are summarized in Table 8. It has been reported that a reduced thiol such as L-cysteine, L-cysteamine or glutathione is required for the release of •NO from certain •NO donor agents such as those containing a furoxan (1,2,5-oxadiazole-2-oxide) moiety.[344] The % •NO release data acquired in this study is consistent with this literature precedent since the % •NO released from the 3,4-diphenylfuroxan compounds (**81-84**) was higher upon incubation in the presence of L-cysteine (0.57 to 3.18%) compared to that determined in phosphate buffer solution at pH 7.4 (0.06 to 0.15%).

Table 6: Nitric oxide release from 3,4-diphenylfuroxans possessing a C-4 H (**81**), SO_2Me (**82a-82b**), SO_2NH_2 (**83**) or SO_2N_3 (**84**) substituent.

	% •NO release[a]	
Compd	**PBS[b]**	**L-cysteine[c]**
81	0.15	0.57
82a-82b	0.06	1.48
83	0.10	0.84
84	0.08	3.18
38[d]	0.72	5.86

[a] Percent of nitric oxide released (mean value, n = 3) quantified as nitrite using the Griess reaction, relative to a theoretical maximum release of 1 mol of •NO / mol of test compound. Variation from the mean % value was ≤ 0.02%.

[b] Incubated in phosphate buffer solution (PBS, pH 7.4) at 37 °C for 1.5 h.

[c] Incubated in the presence of 5 mM L-cysteine in phosphate buffer solution (pH 7.4) at 37 °C for 1.5 h.

[d] The percent nitric oxide released was estimated as the % •NO produced / nitrooxy (ONO_2) group present in the reference drug glycerine trinitrate (**38**, Figure 16).

In comparison, the reference drug glycerine trinitrate released 5.86 % •NO upon incubation in the presence of L-cysteine, and 0.72% •NO in phosphate buffer at pH 7.4, per nitrooxy group. Antiinflammatory agents releasing •NO that reversibly activate soluble guanylate cyclase which in turn catalyze the conversion of guanosine triphosphate to cyclic guanosine monophosphate (cGMP)[208] would be expected to relax smooth muscle in blood vessels, inhibit platelet aggregation and adhesion, and block the adhesion of white cells to blood vessel walls.[209] Accordingly, hybrid COX-2 inhibitor / •NO donor agents should not cause adverse cardiovascular events such as an increased incidence of heart attacks and strokes.

The binding interactions of the 3,4-diphenyl-1,2,5-oxadiazole-2-oxide (3,4-diphenylfuroxan) regioisomers (**82a-82b**, COX-2 IC_{50} = 0.12 μM; COX-2 S.I. = 96) within the COX-2 binding site were examined by molecular modeling (docking) experiments (Figure 40 and Figure 41). The most stable conformer of the regioisomer **82a** orients within the COX-2 primary binding site such that C-4 *para*-methanesulfonylphenyl substituent is positioned in the vicinity of the COX-2 secondary pocket where it is surrounded by amino acid residues Phe^{518}, Arg^{513}, Gln^{192}, Val^{523} and Leu^{352} (Figure 40). One of the *O*-atoms of the SO_2Me substituent forms a favorable hydrogen bond with the backbone N*H* of Ile^{517} (distance = 2.22 Å) and a weak hydrogen bond with the NH_2 of Gln^{192} (distance = 3.62 Å). The distance between the second *O*-atom of SO_2Me and the NH_2 of Arg^{513} was about 7.58 Å. The unsubstitued C-3 phenyl ring was oriented towards the top of the COX-2 binding site closer to Trp^{387} and Tyr^{385}. The N^5-atom of the central furoxan ring participates in a hydrogen bonding interaction with the O*H* of Tyr^{355} (distance = 3.16 Å) and it is positioned about 4.25 Å away from the NH_2 of Arg^{120} that is located near the mouth of the COX-2 binding site. Accordingly, the *O*-atom of the central furoxan ring undergoes a weak hydrogen bonding interaction with the O*H* of Tyr^{355} (distance = 3.63 Å) and it is located about 5.4 Å from the NH_2 of Arg^{120}. It is interesting to note that the *N*-oxide moiety of the central furoxan ring is oriented close to Ser^{530}, the acetylation site of aspirin. The distance between negatively charged N^2-oxido *O*-atom and the O*H* of Ser^{530} was about 2.5 Å. This observation is consistent with previous studies where the C=*O* of the central furanone ring of rofecoxib undergoes a favourable hydrogen bonding interaction with the O*H* of Ser^{530}.[350]

A similar molecular modeling experiment where the regioisomer **82b** was docked in the COX-2 binding site shows, like that observed for **82a**, that the C-3 phenyl ring possessing the *p*-SO_2Me COX-2 pharmacophore is also oriented in the vicinity of the COX-2 secondary pocket (Phe^{518}, Arg^{513}, Gln^{192}, Val^{523}, Ser^{353}, and Leu^{352}) as shown in Figure 41. One of the *O*-atoms of the -SO_2Me substituent is hydrogen bonding with the backbone N*H* of Ile^{517} (distance = 2.28 Å) and the NH_2 of Gln^{192} (distance = 2.08 Å). The C-4 unsubstituted phenyl ring present in **82b**, similar to that observed for regioisomer **82a,** is also oriented towards a hydrophobic area comprised of Trp^{387} and Tyr^{385} at the top of the COX-2 binding site. The major difference between the binding modes observed for the two regioisomers **82a** and **82b** within the COX-2 binding site is the orientation of the *N*-oxido moiety of the central furoxan ring. In the case of regioisomer **82b**, the *N*-oxido moiety is oriented towards the mouth of the COX-2 binding site close to Tyr^{355} and Arg^{120}. In contrast, the *N*-oxido moiety present in **82a** was oriented in a direction close to Ser^{530}. The distance between the negatively charged *O*-atom of the *N*-oxido moiety in **82b** and the O*H* of Tyr^{355} is about 3.3 Å, whereas the distance between the charged guanidino side chain of Arg^{120} and the *O*-atom of the *N*-oxido moiety is about 6.0 Å. Molecular dynamics (MD) simulations on the stabilities of the enzyme-ligand complexes revealed that **82a** ($E_{\text{intermolecular}}$ = -52.30 kcal/mol) has a slightly higher binding affinity for the COX-2 isozyme as compared to **82b** ($E_{\text{intermolecular}}$ = -52.10 kcal/mol).

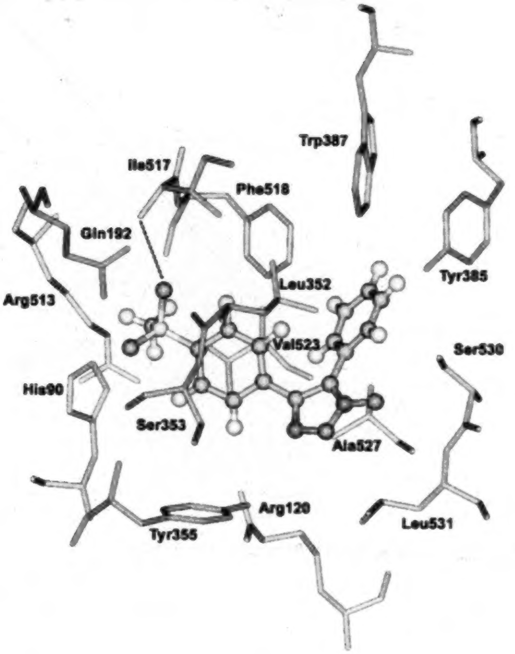

Trp387
Ile517
Phe518
Gln192
Arg513
Leu352
Tyr385
Val523
Ser530
His90
Ser353
Ala527
Arg120
Tyr355
Leu531

Figure 40: Docking of 4-[4-(methylsulfonyl)phenyl]-3-phenyl-1,2,5-oxadiazole-2-oxide (**82a**) (ball-and-stick) in the active site of murine COX-2. Hydrogen atoms of the amino acid residues have been removed to improve clarity.

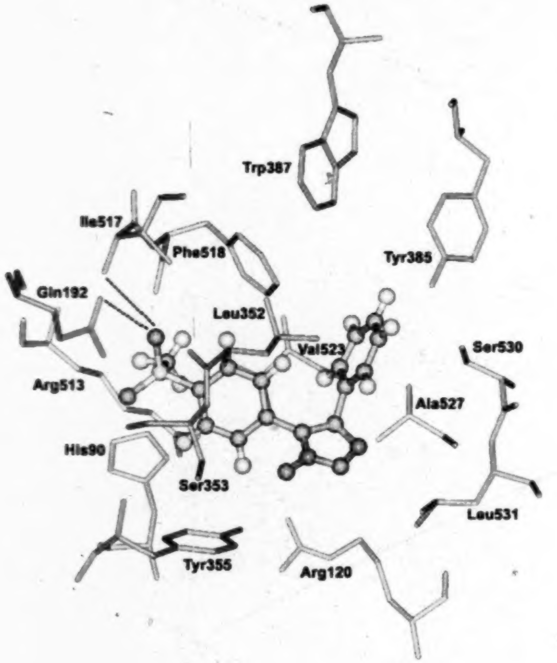

Trp387
Ile517
Phe518
Tyr385
Gln192
Leu352
Val523
Ser530
Arg513
Ala527
His90
Ser353
Leu531
Tyr355
Arg120

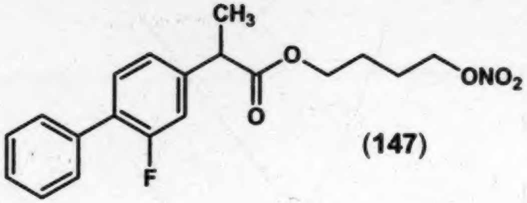
Figure 41: Docking of 3-[4-(methylsulfonyl)phenyl]-4-phenyl-1,2,5-oxadiazole-2-oxide (**82b**) (ball-and-stick) in the active site of murine COX-2. Hydrogen atoms of the amino acid residues have been removed to improve clarity.

4.2.4. Project 4: •NO-NSAIDs (89-94).

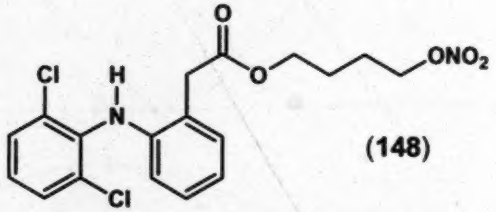
4.2.4.1. Preamble.

Chronic use of NSAIDs is associated with alterations in gastrointestinal integrity and function[129,130] which results in the development of gastric ulcers.[325] Thus, the gastric irritant effect of aspirin (**17**) can be a deterrent to its long-term use for the prophylactic prevention of adverse cardiovascular events such as stroke and myocardial infarction.[326] Aspirin is a unique nonselective COX inhibitor due to its ability to acetylate the Ser^{530} hydroxyl group in the primary COX binding site of COX-1 and COX-2. In this regard, aspirin is a 10- to 100-fold more potent inhibitor of COX-1 relative to COX-2.[351] Acetylation of the weakly nucleophilic OH of Ser^{530} by aspirin is thought to result from initial binding of its COOH to Arg^{120} near the mouth of the COX binding site, which positions the ortho-acetoxy moiety in close proximity to the Ser^{530} OH, which it acetylates. Orally administered aspirin irreversibly acetylates Ser^{530} of COX-1 in platelets,[163] which results in a complete inhibition of platelet-derived thromboxane A_2 (TxA_2) biosynthesis. TxA_2 is a potent platelet aggregator which also induces vasoconstriction and smooth muscle proliferation.[161] However, there remains a significant risk of gastrointestinal bleeding[275] due to inhibition of COX-1-mediated gastric PG synthesis even with low prophylactic doses of aspirin.[135]

It has been proposed that the linking of an •NO-releasing moiety to an NSAID may reduce the toxicity of the latter.[247] In animal studies, •NO-releasing derivatives of a wide range of NSAIDs including the •NO-aspirin (**64**, Figure 23 page 48), •NO-naproxen (**65**, Figure 24 page 49), •NO-flurbiprofen (**147**) and •NO-diclofenac (**148**) (Figure 42), have been shown to spare the gastrointestinal tract, even though they suppressed prostaglandin synthesis as effectively as the parent drug.[247,248,255]



(147)



(148)

Figure 42: Chemical structures of •NO-flurbiprofen (**147**) and •NO-diclofenac (**148**).

All these •NO-releasing NSAIDs have a nitrooxyalkyl group as the •NO-releasing group. However, an important drawback to this design is the fact that production of •NO from organic nitrate esters requires a three-electron reduction, and this metabolic activation decreases in efficiency on continued use of the drugs, contributing to "nitrate tolerance".[324] In this regard, O^2-unsubstituted *N*-diazen-1-ium-1,2-diolates have the potential to release up to 2 equivalents of •NO with half-lives that correlate well with their pharmacological durations of action. These observations suggest that *N*-diazen-1-ium-1,2-diolates are minimally affected by metabolism, and are essentially different from currently available clinical vasodilators that require redox-activation before •NO is released.[235]

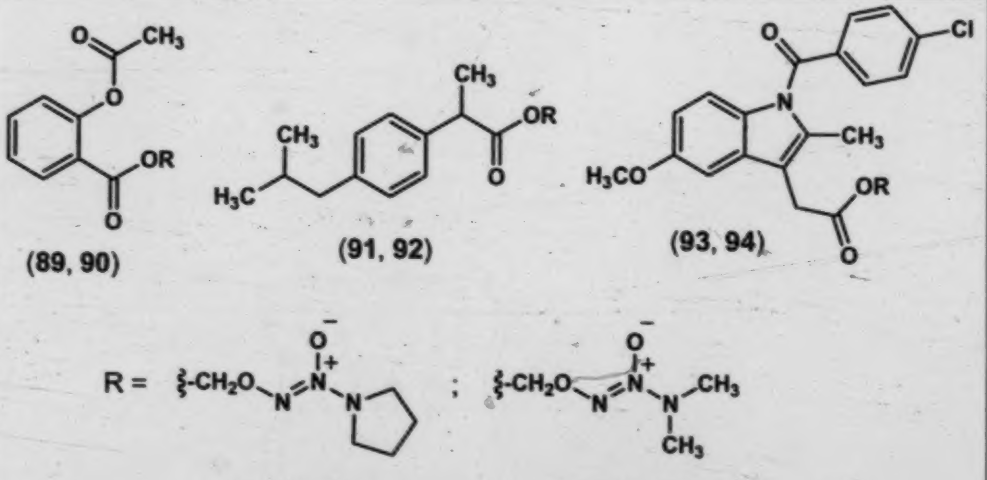
4.2.4.2. Results and discussion.

A group of new •NO-releasing non-steroidal antiinflammatory drugs (•NO-NSAIDs), derived from aspirin (**89**, **90**), ibuprofen (**91**, **92**) and indomethacin (**93**, **94**) possessing a 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate, or 1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate moiety were synthesized. *In vitro* COX enzyme inhibition studies (Table 9) showed that none of these compounds inhibited either the COX-1 or COX-2 isozyme at the highest test compound concentration used (100 μM).

Thus, attachment of an ester group (the •NO-releasing diazeniumdiolate moiety) to the parent NSAID completely abolished the *in vitro* enzyme inhibitory activity of aspirin, ibuprofen and indomethacin. However, when administered orally to rats, the carrageenan-induced rat paw edema assay (Table 9) provided similar ID_{50} values to those obtained for the reference drugs. The ibuprofen •NO-NSAIDs **91** and **92** showed equipotent antiinflammatory activities (ID_{50} = 66.8 and 62.3 mg/kg respectively) compared to the reference drug ibuprofen (ID_{50} = 67.4 mg/kg). Similar results were obtained for the •NO-aspirins **89** (ID_{50} = 181.8 mg/kg) and **90** (ID_{50} = 151.2 mg/kg), and the •NO-indomethacin **94** (ID_{50} = 5.9 mg/kg), which were 1.1-1.4-fold less potent relative to the parent drugs aspirin (ID_{50} = 128.7 mg/kg) and indomethacin (ID_{50} = 4.2 mg/kg). In comparison, the •NO-indomethacin **93** (ID_{50} = 10.7 mg/kg) was about 2.5 fold-less potent than indomethacin.

Compounds containing a 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (**89**, **91** and **93**) moiety were less active than those compounds having a 1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate moiety (**90**, **92** and **94**). It has been reported that aspirin acetylates the Ser^{530} residue in the COX-1 active site. The observations that both •NO-aspirins (**89** and **90**) were inactive *in vitro* inhibitors of COX-1 and COX-2 (IC_{50} > 100 μM), and that they showed significant antiinflammatory activities *in vivo*, strongly suggests that **89** and **90** act as classical prodrugs, which require a metabolic activation reaction (esterase-mediated ester cleavage) to be active.

Table 7: *In vitro* COX-1/COX-2 enzyme inhibition, and *in vivo* antiinflammatory activity data for •NO-NSAIDs **89-94**.



(89, 90) (91, 92) (93, 94)

R =

(89, 91, 93) (90, 92, 94)

Compd.	COX-1 IC$_{50}$ (μM)[a]	COX-2 IC$_{50}$ (μM)[a]	COX-2 S.I.[b]	AI activity[c] ID$_{50}$ (mg/kg)
89	> 100	> 100	-	181.8
90	> 100	> 100	-	151.2
91	> 100	> 100	-	66.8
92	> 100	> 100	-	62.3
93	> 100	> 100	-	10.7
94	> 100	> 100	-	5.9
Aspirin	0.3	2.4	0.14	128.7
Ibuprofen	2.9	1.1	2.63	67.4
Indomethacin	0.1	5.7	0.01	4.2

[a] The *in vitro* test compound concentration required to produce 50% inhibition of COX-1 or COX-2. The result (IC$_{50}$, μM) is the mean of two determinations acquired using an ovine COX-1/COX-2 assay kit (Catalog No. 560101, Cayman Chemicals Inc., Ann Arbor, MI, USA) and the deviation from the mean is <10% of the mean value.

[b]Selectivity index (SI) = COX-1 IC_{50}/COX-2 IC_{50}.

[c]Inhibitory activity in a carrageenan-induced rat paw edema assay. The results are expressed as the ID_{50} value (mg/kg) at 3 h after oral administration of the test compound.

One type of chemical modification used to control the rate of •NO release from diazen-1-ium-1,2-diolates is the attachment of alkyl substituents to the O^2-position. O^2-substituted-diazen-1-ium-1,2-diolates are stable compounds that hydrolyze slowly even in acidic solution. Consistent with these observations, when compounds **89-94** were incubated in PBS at pH 7.4, the percentage of •NO released varied from 14.3 to 16.1 % which is indicative of slow •NO release. In contrast to recently reported O^2-acetoxymethyl-1-(pyrrolidin-1-yl or *N,N*-diethylamino)diazen-1-ium-1,2-diolates,[240] which are stable prodrugs in neutral aqueous media but which released about 1.8 equivalents of •NO (> 90% release) per mol of drug upon metabolism by porcine liver esterase (PLE), the ester prodrugs **89-94** are hydrolyzed much less extensively (16.3 to 19.2% •NO release). However, the effect of non-specific esterases present in guinea pig serum on the •NO release properties of compounds **89-94** was substantially higher (81.6-93.6% range) than that observed (16.3-19.2% range) upon incubation with PLE (see Table 10).

These data indicate the non-specific serum esterases present in guinea pig serum cleave these •NO-NSAIDs more effectively than PLE.

Table 8: Nitric oxide release for •NO-NSAIDs **89-94**.

Compd	% of Nitric oxide released[a]		
	PBS (pH 7.4)[b]	PLE[c]	GP-Serum[d]
89	14.8 ± 0.1	18.5 ± 0.1	88.9 ± 0.2
90	15.4 ± 0.1	19.1 ± 0.1	81.6 ± 0.1
91	14.9 ± 0.1	16.3 ± 0.1	89.2 ± 0.1
92	16.1 ± 0.1	17.3 ± 0.1	93.6 ± 0.1
93	15.1 ± 0.1	16.3 ± 0.1	89.1 ± 0.1
94	14.3 ± 0.1	16.9 ± 0.1	86.3 ± 0.1
115	95.2 ± 0.1	-	-
57	94.0 ± 0.1	-	-

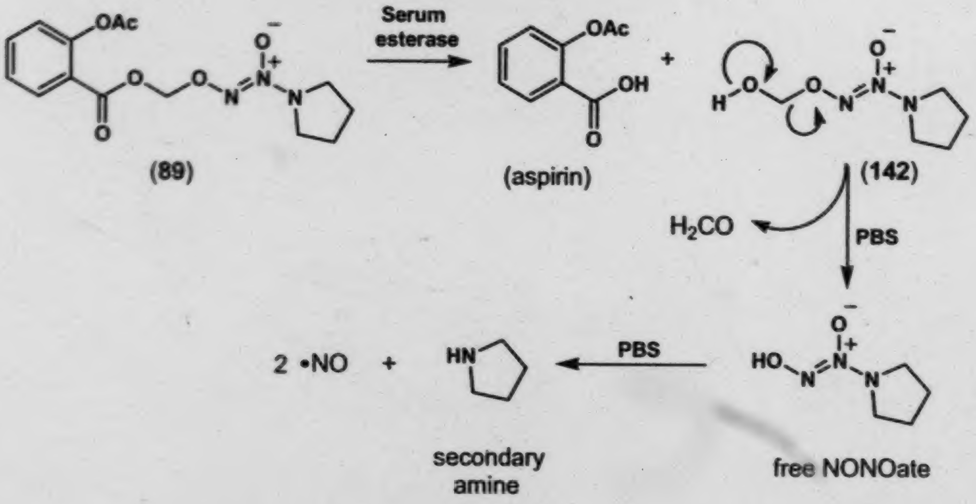
[a] Percent of nitric oxide released (± SEM, n = 3) quantified as nitrite using the Griess reaction, relative to a theoretical maximum release of 2 mol of •NO/mol of test compound.

[b] Incubated in phosphate buffer solution (PBS, pH 7.4) at 37 °C for 1.5 h.

[c] Incubated in the presence of 2 equivalents of pig liver esterase (based on a ratio of 1 mol of test compound / 2 mol of esterase) in phosphate buffer solution (pH 7.4) at 37 °C for 1.5 h.

[d] Test compound (2.0×10^{-4} mmol) incubated with guinea pig serum (260 μL) in phosphate buffer solution (pH 7.4) at 37 °C for 1.5 h.

Although conventional •NO donors can protect the stomach against NSAID-induced gastric damage, they do not do so as effectively as NSAIDs (including aspirin) that are chemically linked to an •NO-releasing moiety.[314] A plausible mechanism for the hydrolysis of these •NO-NSAID ester prodrugs **89-94** is presented in Scheme 19. The •NO-NSAID ester prodrugs **89-94** were designed with a one-carbon methylene spacer between the carboxy group and the diazen-1-ium-1,2-diolate O^2-atom, such that the O^2-(hydroxymethyl)diazen-1-ium-1,2-diolate (**142**) compound formed after ester cleavage would spontaneously eliminate formaldehyde to produce the free NONOate compound that can subsequently fragment to release two molecules of •NO.



Scheme 19: Theoretical metabolic activation (hydrolysis) of •NO-NSAIDs (compound **89** is shown as a representative example).

One of the common side effects of NSAID therapy is gastrointestinal irritation and bleeding. It was therefore essential to evaluate the prodrugs **89-94** ulcerogenicity in comparison to that induced by the three parent drugs. The severity of gastric damage was expressed as an ulcer index (Table 11). There was a remarkable difference between the ulcer index values for the •NO-NSAIDs (UI = 0-3.0), and the reference drugs aspirin (UI = 57.4, 250 mg/kg po dose), ibuprofen (UI = 45.7, 250 mg/kg po dose) and indomethacin (34.4, 30 mg/kg po dose). This UI data suggests a safer pharmacological profile for hybrid •NO-NSAIDs containing either a 1-(pyrrolidin-1-yl or *N,N*-dimethylamino)diazen-1-ium-1,2-diolate group, relative to the parent drugs. No evidence of gastric ulcerogenicity (UI = 0) was observed (Figure 43 and Figure 44) for either the •NO-aspirin (**89**, **90**) and •NO-ibuprofen (**91**, **92**) ester prodrugs. The •NO-indomethacin compounds (**93**, **94**) caused minimal ulcerogenicity (UI = 0.7-3.0 range) (Figure 45).

Table 9: Gastric ulcer index produced by acute administration of a single dose of the test compounds **89-94** and the reference drugs aspirin (**17**), ibuprofen (**20**) and indomethacin (**22**).

Compd.	Ulcer index[a]
aspirin	57.4 ± 3.1[b]
ibuprofen	45.8 ± 2.9[b]
indomethacin	34.4 ± 4.2[c]
89	0[d]
90	0[d]
91	0[e]
92	0[e]
93	0.7 ± 0.11[f]
94	3.0 ± 0.3[f]
control group	0[g]

[a] The average overall length (in mm) of individual ulcers in each stomach ± SEM, n = 4, at 6 h after oral administration of the test compound.

[b] 250 mg/kg dose.

[c] 30 mg/kg dose.

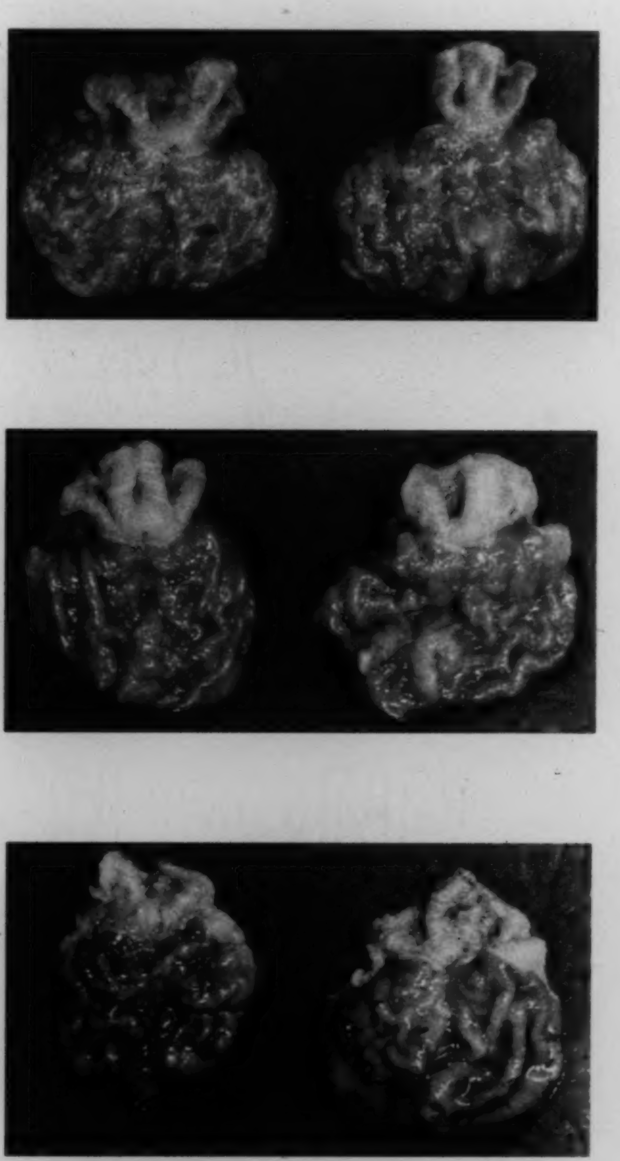
[d] Equivalent amount to 250 mg of aspirin/kg.

[e] Equivalent amount to 250 mg of ibuprofen/kg.

[f] Equivalent amount to 30 mg of indomethacin/kg.

[g] 1.0% methylcellulose solution.

Figure 43: Ulcerogenicity assay data illustrating the extent of NSAID-induced gastric ulcers for •NO-NSAID **89**, compared to that induced by the parent drug aspirin (**17**).



Control group

•NO-aspirin (**89**)

aspirin (**17**)

Figure 44: Ulcerogenicity assay data illustrating the extent of NSAID-induced gastric ulcers for •NO-NSAID **91**, compared to that induced by the parent drug ibuprofen (**20**).

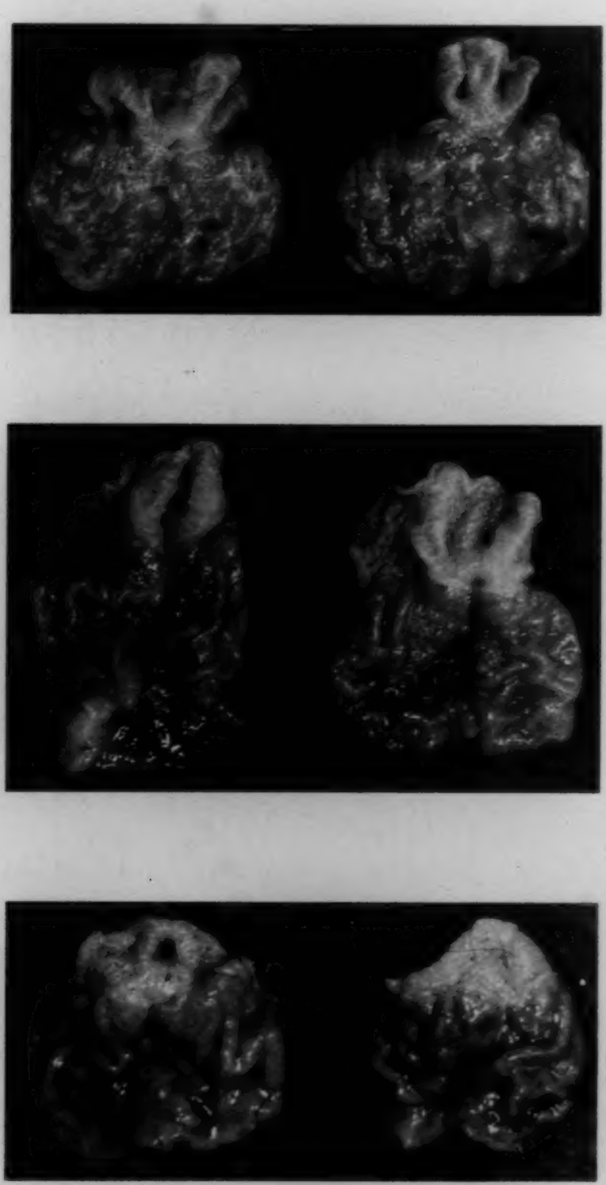
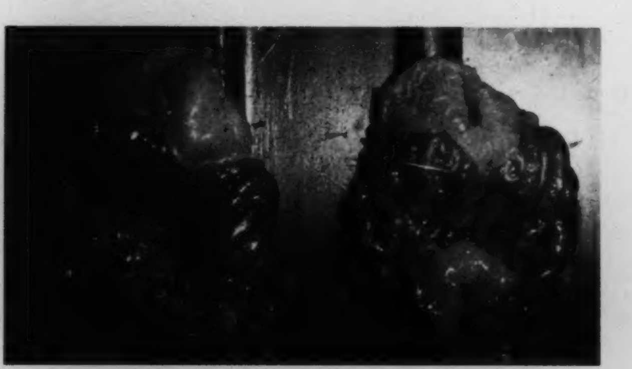

Control group
•NO-ibuprofen (**91**)
ibuprofen (**20**)

Figure 45: Ulcerogenicity assay data illustrating the extent of NSAID-induced gastric ulcers for •NO-NSAID **93**, compared to that induced by the parent drug indomethacin (**22**).



Control group

•NO-indomethacin (**93**)

indomethacin (**22**)

5.0. Conclusions

A group of racemic 4-aryl(heteroaryl)-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylates possessing a potential •NO donor C-5 O^2-alkyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate ester [alkyl = $(CH_2)_n$, n = 1-4] substituent were successfully synthesized using a modified Hantzsch reaction. Compounds having a C-4 benzofurazan-4-yl (**67-70**) substituent exhibited weaker smooth muscle calcium channel antagonist activity (IC_{50}'s in the 0.55 to 8.46 μM range), relative to the reference drug nifedipine (IC_{50} = 0.0143 μM). The alkyl spacer groups of variable chain length [$-CO_2(CH_2)_nO-$, n = 1-4] exhibited small differences in calcium channel antagonist potency.

Replacement of the ester "isopropyl" group present in the C-4 benzofurazan-4-yl group of compounds by an O^2-alkyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate ester substituent provided compounds **67-70** (n = 1 and 4) that were approximately equipotent cardiac positive inotropes (IC_{50}'s in the 2.45 – 6.25 μM range) with the parent reference compound PN 202-791 (**12**, EC_{50} = 9.40 μM).

The O^2-alkyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate ester moiety present in 1,4-dihydropyridine calcium channel modulating compounds **67-70** is not a suitable •NO donor moiety since the percent nitric oxide released upon in vitro incubation with either L-cysteine, rat serum or pig liver esterase was less than 1%. Despite the fact that compounds **67-70** did not hydrolyze as originally anticipated, they still showed moderate calcium channel modulation activities.

The group of racemic 4-aryl(heteroaryl)-1,4-dihydro-2,6-dimethyl-3-nitropyridine-5-carboxylates possessing a •NO donor O^2-acetoxymethyl-1-(*N*-ethyl-*N*-methylamino)diazen-1-ium-1,2-diolate (**71-75**) or O^2-acetoxymethyl-1-(4-ethylpiperazin-1-yl)diazen-1-ium-1,2-diolate (**76-80**) C-5 ester substituent, constitute a novel group of nitric oxide donor compounds with desirable calcium channel modulation activities. In particular, compounds having a C-4 2-trifluoromethylphenyl substituent (**74** and **79**) exhibit *dual cardioselective agonist/smooth muscle selective antagonist activities*, in conjunction with an *enhanced nitric oxide release profile* compared with other nitric oxide functional group donors reported in the literature, such as furoxans.[31,32] Drugs having these desirable tissue selective CC modulation properties offer potential for the treatment of CHF.

The replacement of the methyl ester moiety of Bay K 8644 by an O^2-acetoxymethyl-1-(*N*-ethyl-*N*-methylamino)diazen-1-ium-1,2-diolate group (**75**), or an O^2-acetoxymethyl-1-(4-ethylpiperazin-1-yl)diazen-1-ium-1,2-diolate group (**80**), retained the desired cardiac positive inotropic effect while abolishing the smooth muscle agonist effect on guinea pig ileum.

Remarkably, the O^2-acetoxymethyl-1-(*N*-ethyl-*N*-methylamino)diazen-1-ium-1,2-diolate and O^2-acetoxymethyl-1-(4-ethylpiperazin-1-yl)diazen-1-ium-1,2-diolate C-5 ester substituents, released up to 3-fold higher amounts of nitric oxide in the presence of *serum esterases*, compared with •NO release in the presence of a liver esterase. This *selective* and *enhanced* •NO release might provide a useful therapeutic advantage towards the treatment of cardiovascular diseases, including congestive heart failure, because it may be possible to increase endothelial •NO with only minor effects at other sensitive sites throughout the organism.

Replacement of the 2-(5*H*)furanone central ring present in rofecoxib by an isosteric 1,2,5-oxadiazole-2-oxide ring maintains COX-2 inhibitory activity, although there is a decrease in COX-2 selectivity, relative to the reference compounds rofecoxib (**23**) and celecoxib (**24**). Structure-activity data acquired for the 3,4-diphenyl-1,2,5-oxadiazole-2-oxides (3,4-diphenylfuroxans) and 3,4-diphenyl-1,2,5-oxadiazoles (3,4-diphenylfurazans) investigated indicate that a methylsulfonyl (SO_2Me), or aminosulfonyl (SO_2NH_2), COX-2 pharmacophore located at the *para*-position of either a C-3 or C-4 phenyl ring is essential for COX-2 inhibitory activity. In contrast, compounds having an unsubstituted C-3 or C-4 phenyl substituent (**81**, **85**) were selective COX-1 inhibitors. The sulfonylazido (SO_2N_3) substituent is not a suitable COX pharmacophore since compounds possessing this substituent were devoid of both COX-1 and COX-2 inhibitory activity.

A central ring *N*-oxido substituent is not a major determinant of COX inhibitory potency and/or selectivity. In this regard, the presence of a *N*-oxido moiety provided small increases in COX-2 potency and selectivity. Alternatively, removal of the *N*-oxido oxygen atom, which gives rise to the *N*-deoxy derivatives, results in a small increase in COX-1 potency and a small decrease in COX-2 potency. The thiol-dependent release of •NO, in conjunction with relatively potent and selective COX-2 inhibitory activity, suggest that the 1,2,5-oxadiazole-2-oxide (furoxan) ring system possesses beneficial features that would be desirable for the design of hybrid COX-2 inhibitor / •NO donor antiarthritic agents with a low ulcerogencity profile and minimal potential to induce adverse cardiovascular events such as heart attacks and strokes.

Hybrid •NO-NSAID ester prodrugs possessing a 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (**89, 91, 93**) or 1-(*N,N*-dimethylamino)diazen-1-ium-1,2-diolate (**90, 92, 94**), moiety attached via a one-carbon methylene spacer to the carboxylic acid group of traditional NSAIDs constitutes a new concept for the rational design of antiinflammatory drugs with reduced gastric side effects (ulcerogenicity). Virtually every NSAID having a free carboxylic acid is suitable for application of this methodology. *In vivo* activation (hydrolysis) of these •NO-NSAIDs by plasma esterases, rather than liver esterases, would be expected to improve the •NO release profile compared to that observed for organic nitrates which require a more metabolically demanding three-electron reduction for the release of •NO, or a thiol cofactor such as L-cysteine or glutathione required for the release of •NO from furoxans.

Hybrid •NO-aspirins having a diazen-1-ium-1,2-diolate moiety could be a useful alternative to the use of aspirin as an antithrombotic agent (inhibition of platelet aggregation) in the long-term prophylactic prevention of stroke and myocardial infarction.

Aspirin is frequently given to patients who are undergoing angioplasty, but it has only modest therapeutic effects on restenosis, and carries the risk of GI ulceration and bleeding mentioned previously. The •NO-aspirins possessing a diazeniumdiolate as the •NO donor moiety, could represent a useful alternative to prevent restenosis, a medical problem associated with percutaneous transluminal coronary angioplasty (Figure 26, page 52) the most widely used treatment for atherosclerosis.[279] Restenosis has been suggested to occur as a consequence of impaired •NO production by the damaged endothelium.[305]

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